

Poor show for aviation industry

This week's Farnborough show of the aircraft industry is not so much a trade fair as a reminder that the industry's chief product, civil air transport, is not as accessible or cheap as it should be.

THE English place-name Farnborough means two things — a research establishment (the Royal Aircraft Research Establishment) and an air show (mounted every other year on the establishment's private airfield by the Society of British Aircraft Constructors). Once, the air show was what the name suggests, an opportunity for the wider public to see and inspect the latest wonders of aviation — aircraft with six wings and then with two; aircraft with metal wings and enclosed cockpits; aircraft capable of carrying passengers; and so on. Now, as this week's show will have amply demonstrated, the biannual show is nothing of the kind. Most of the exhibitors are not plane-makers but would-be manufacturers of bits and pieces from which aircraft may eventually be assembled. Those who make navigation equipment, or who provide fast-food for airline passengers, may even turn out to have done enough business at this year's show to justify the cost of being present. But in spite of the ritual flying display by familiar flying machines, most of them military, the show should be, for most of those who attend it, a reminder that the aircraft business is in a mess. And that it will remain so unless drastic steps are taken towards reform.

The problems are mostly political, deriving from the way in which governments of most countries seek short-term gains from technology at the expense of the potential beneficiaries — ordinary people, either as air-travellers or as taxpayers. Since the days when biplanes were made robust enough reliably to cross the narrow strip of water between England and France during the First World War, the aircraft business has become thoroughly international in the particular sense that international travel has become habitual and largely beneficial. Families split by emigration half a century ago unexpectedly find themselves reunited. People who choose to work in two continents can do so. People bored with vacations at Morecambe or Martha's Vineyard can hope to swim off Marrakesh instead. Even though groups of malcontents occasionally seize civil aircraft and force them to fly to places not on their flight-plans, the general influence of civil air transport is rightly to be welcomed. The tragedy is that the benefits of this important technology would be even more widely shared if it were properly exploited.

Problems

There are three ingredients of the muddle afflicting aircraft technology, all of them familiar. The chief is the practice by which most governments connive at the distortion of the air travel market by subsidizing national airlines and then by seeking to minimize the costs of that malpractice by bartering the right to fly over international routes with other governments. Second is the argument, even by governments guiltless on the first count, that aircraft technology is so closely bound up with military security that self-sufficiency is essential. Finally, the cost of developing new flying machines has been allowed, by lax governments and extravagant manufacturers, to become so large that the cost of equipment — and the risks in making it — are needlessly great. This is how governments and manufacturers should set out to put this ramshackle industry in order.

The distortion in the market for air travel is sanctified by an international conference in Ottawa in 1944, in the heady days when people hoped the United Nations would replace chauvinism by international charity. For much of the past 40 years, the

International Air Transport Authority (IATA) which was a direct result has functioned as a simple cartel, allowing airlines to fix the prices charged for tickets on international routes. The same experience, working on the principle that the slice of the lower atmosphere above each country is part of its sovereign territory, launched the iniquitous doctrine that governments should be required to negotiate bilaterally how many flights by which airlines there should be between one and the other. In most parts of the world, the result has been that prices have been too high and services too few. That there would be problems in the outright deregulation of international air transport is not denied. Maverick governments willing indefinitely to support their national airlines could easily drive into bankruptcy competing airlines enjoying reciprocal rights but organized commercially. It would simply be necessary to drive down fares. The short-term remedy is the scheme the British and Dutch Governments have imaginatively drawn up for deregulating traffic between London and Amsterdam — there is a low minimum fare, but restrictions on frequency and the onward destinations of passengers are abolished. Other governments should follow suit — and in Europe, the European Commission should prod them more vigorously. Further ahead, there is a crying need that all airlines should operate on near-commercial principles. In Europe, the best solution would be a European fund specially created to finance airline operations in the inevitable years of deficit — and an agreement that airlines that cannot survive prudent bankers' investigation should go to the wall.

Chauvinism

The chauvinism that runs through the aircraft manufacturing business with disastrous consequences must somehow be moderated. Outside the Soviet Union and China, there are only three practical suppliers of large civil aircraft (Boeing, McDonnell-Douglas and Airbus Industrie, the European consortium) and three manufacturers of large jet engines (Rolls-Royce, General Electric and Pratt and Whitney). This is probably as many as a thin market will support, yet these companies are increasingly under pressure to make deals with local manufacturers in the countries whose national airlines agree to buy their wares, sometimes to abate the purchase price, more often to acquire unfamiliar skills. In military aircraft, the consequences of chauvinism are even worse. The widespread practice, even among military allies, of manufacturing as much as possible of their service aircraft is a means of increasing the great cost of these machines, and of keeping together groups of workers which are already too large. The excuse is that indigenous technology and manufacturing capacity are strategic necessities, a particularly thin excuse when military planners say that future wars will be unpleasantly nasty but mercilessly short. The economic consequences of these arrangements are easily guessed. Modern aircraft are assembled from bits and pieces themselves manufactured by companies which are smaller than they need be, and often too small to be fully skilled. Flying the flag, as the saying goes, is a way of making aircraft more expensive, and therefore less useful, than they might be.

The technical extravagance of the major manufacturers tends in the same direction. The unit of accounting in the development of a new aircraft or of its engines seems to have settled down at



£1,000 million or thereabouts. (Boeing's past successes may owe something to its economical ways of working.) Even allowing for the technical sophistication of the new machines and their power plants, and for the value of the potential prizes to be won, it is hard to see how costs on this scale can be justified. Part of the explanation is the ambition of most manufacturers to keep in being the small armies of skilled people which constitute their design teams. It would do no harm, and indeed might even benefit the aircraft industry itself, if the wisdom of these practices were closely examined by people skilled at telling not merely whether carbon-fibre composites are better for some technical purpose than the new aluminium-lithium alloys, but whether the processes of design and development need be as expensive as they have become. The economic benefits could be substantial — more flexibility in too tightly hierarchical an industry, easier access by the people to a technology whose benefits are already great, but which could be huge. Governments will not attempt the hard-eyed scrutiny that the aircraft industry needs, but would undoubtedly welcome the results. The responsibility falls squarely on the technical community. Why not (for Britain) Farnborough? □

Too brief encounter

The likely outcome of the space weapons talks is that the negotiators will meet only to adjourn.

THOSE who believe diplomacy a dull business should think again. Some weeks ago, the Soviet Union invited — some would say challenged — the United States to a conference at Vienna on the use of the space above the atmosphere for the siting of military weapons. The date suggested for the opening of the conference is 18 September, less than two weeks from now. Yet as things were earlier this week, nobody knew for certain whether two small armies of diplomats would actually arrive, ready to begin talking to each other. Careful people that they are, the potential members of the negotiating teams have arranged for hotel rooms to be booked on their behalf.

But Mr Konstantin Chernenko is clearly a little anxious that nothing at all will happen. In his interview with *Pravda* published at the weekend, he made noises apparently designed to soften the quarrel there has been with the United States about the agenda for the talks. If, he suggested, the United States would agree to talk about space weapons only at Vienna, he might ensure that there would be negotiations at another place and time about the issues the United States says it must discuss — the disposal of nuclear weapons in Europe and more broadly. Unfortunately, Mr Chernenko seems to have reckoned without the American calendar, temporal and electoral. Last weekend was Labor Day weekend, when citizens of the United States do not labour. And with President Ronald Reagan off to a good start in his campaign for re-election only two months from now, giving hostages to fortune in a negotiation that could not be complete before election day was bound to go against the electoral gain. So the State Department quickly announced that there was nothing new in what Mr Chernenko had to say, even though it is clear that it provided an opportunity that could have been exploited if anybody had felt so inclined.

What happens next? The most serious danger is that the preoccupation of even enlightened spirits in the United States with the election will blind one partner in the talks to the importance of what needs doing. Meanwhile, even the President and his advisers might prudently calculate (as they should have done during the last election campaign) that talks with the Soviet Union will be necessary once the dust has settled. Just as well to get something started before the election, but not to get too deeply embroiled in negotiations until the polls have taken place. So the likely calculation, at the State Department, is to aim at a preliminary encounter over space weapons, a polite statement of the need to talk about other things as well and an offer to adjourn the impending negotiation until something can be arranged. Whether Mr Chernenko and his colleagues will agree is entirely unclear. □

Academic divisions

Polytechnics should be made into universities or otherwise transformed.

THESE are busy but depressing days for polytechnics, the Cinderellas of the British system of higher education. For this is the season of the year at which would-be students denied places at universities turn to the polytechnics in the hope of being fitted in. On past form (and the figures released earlier this week by the Universities' Central Council on Admissions), something like 100,000 applicants for university places will be refused (but 75,000 will be accepted). Those turning to the polytechnics for an undergraduate education will, however, be doused with cold water by the report now published of a study of *Expectations of Higher Education* commissioned in 1980 by the Department of Education and Science and carried out by the Department of Government at Brunel University, the head of which (Professor Maurice Kogan) is as much an educationalist as anything else. The short message of a formless document is simple — employers are biased against the recruitment of graduates from the polytechnics, preferring the products they are used to — graduates from universities and especially from Oxford and Cambridge. This unsurprising news will be a slap in the face for many of the young hopefuls now hurrying off to the public sector in the hope of finding places as undergraduates. For those concerned with the future of British higher education, however, it should be a reminder that present arrangements are far from being sensible, and should urgently be scrapped.

Britain is not unique among industrialized nations in maintaining two kinds of institutions of higher education. Usually there are more vocational educational institutions. The distinctiveness of the British system is that the polytechnics were established overtly as an alternative to the university system, and have ever since been advertised as such. Whether the motives of the minister responsible, the late Mr Anthony Crosland, were political (British universities were already becoming unpopular with politicians), economic (the technical colleges from which the polytechnics emerged were thought to be cheaper) or pragmatic (what else could have so quickly increased the supply of graduates?), the designation of 26 polytechnics as graduating institutions within what is called the binary system turns out to have been a mistake. If there are two classes of institutions, one of which is seen to attract less highly qualified students (and probably less talented teachers), yet which has failed in nearly twenty years to make a characteristic mark, who can be surprised if that one continues to seem second-rate?

The prejudice of employers of all kinds against polytechnic graduates is understandable, even forgivable, on the basis of the data collected by the Kogan study. The school-leaving qualifications of polytechnic students, which span a spectrum overlapping with that of university entrants, are in general and on the average lower than those of new university undergraduates. While any process of education must somehow be capable of improving on people's attainment at the outset of their academic careers, nobody should be surprised that some of these measurable characteristics persist — and that when jobs are hard to find, employers take the easy way out. But all this is unfair to the polytechnics, many of which are in every way at least as competent as fully-fledged universities, and as successful in preparing their students for valuable careers. Especially because of the way that, in Britain, the sharpness of the binary division is accentuated by the academic snobbery which is rife, there is the strongest possible reason for its abolition. The sensible course would be to set a term, say five years from now, when all these institutions would be freed from the apron-strings of the local authorities that now watch over them, some to become rounded institutions of higher education, others to make their way in the world in different ways, perhaps by providing the distinctively vocational higher education that was originally, and inconsistently, part of the reason for their creation. Others might have to sink without trace. □

Biotechnology

Cohen-Boyer patent finally confirmed

Washington

AFTER 10 years of legal argument, Stanford University has finally been granted a "groundbreaking" product patent covering recombinant DNA molecules as produced by the Cohen-Boyer gene cloning technique. The Cohen-Boyer technique is fundamental to most genetic engineering research, and the new patent, which includes the uses of recombinant DNA in bacterial cells, confirms Stanford's proprietary hold on the fledgling biotechnology industry. A process patent covering Cohen-Boyer was granted to the university in 1980, and is considerably strengthened by the new product patent.

At present 66 companies are licensed to use the technology by Stanford, which shares rights with the University of California at San Francisco. Others have been holding back from paying the \$10,000 annual licence fee until the status of the product patent became clear. The university will from now on enforce both, and it is likely that most industrial users of the technique will pay up: as one patent attorney put it, "you'd have to be out of your mind to challenge a \$10,000 licence" since legal costs would inevitably be much greater than the licence fee.

The Cohen-Boyer technique uses a bacterial plasmid to transfer foreign DNA into a host cell. While the cost to licensees of using the technique might be modest, the value of the patents to Stanford and the University of California, San Francisco, is likely to be considerable, especially when genetically engineered products become more widely available. The universities will share royalties at the rate of 1 per cent for the first \$5 million of sales, decreasing to ½ per cent on sales over \$10 million. Use of the Cohen-Boyer technique in pure research is not restricted, but the partners have already earned almost \$3 million on the process patent from commercial companies. The two inventors, Dr Stanley Cohen of Stanford's school of medicine and Dr Herbert Boyer, professor of biochemistry at the University of California, San Francisco, have both waived their rights to a one-third share of revenues. The income is used by the universities to support graduate student programmes. The new patent will prevent, for example, companies avoiding royalty payments by manufacturing abroad and importing into the United States.

The Cohen-Boyer saga has been controversial on several counts. In 1982, the Patent and Trademark Office tentatively rejected the product patent on the grounds that the original application incorrectly

specified the origin of the plasmid called pSC101. The plasmid was described in the application as arising from a naturally occurring plasmid by recircularization of a fragment itself produced by hydrodynamic shear. A later paper by Cohen and a co-author revised this view and left open several possibilities for the plasmid's origin.

A patent specification must under US law provide sufficient information to enable a person "skilled in the art" to reproduce the invention. In granting the new product patent the patent office seems to have recognized that a description of how to make a product contains sufficient disclosure if it is operationally accurate, even if the proposed interpretation is incorrect.

Also related to the disclosure requirement was the objection that Cohen and Boyer failed to deposit samples of their plasmid in a public repository at the time the application was filed. They did, however, make samples available to

qualified investigators, and the patent office seems now to have accepted that this practice was not unduly restrictive. This aspect of the case has caused some consternation to lawyers, who see dangers in allowing an organism in a private repository to suffice as a patent standard. Not least of these dangers is the natural inclination of a scientist to replace a strain isolated for a specific purpose with a better specimen should one turn up.

Other issues raised have included that of whether the invention was non-obvious and whether the scope of the application was too broad. Despite some fleeting references to the possibility of artificially recombining DNA published more than a year before the application, the new ruling confirms the originality of the idea. Stanford has withdrawn an earlier claim over the use of recombinant molecules in eukaryotic cells to expedite the present patent, but is still pursuing that claim in a separate undisclosed application.

The patent office usually declines comment on interpretation of its decisions, although an official said the judgement did "not represent a change of policy". A detailed policy statement on biotechnology is now being prepared by the patent office and is expected to clear up much of the confusion left in the wake of the Cohen-Boyer patent.

Tim Beardsley

Industrial training

Britain lags well behind

A NEW approach to vocational training and education in Britain is essential if British industry is to compete successfully in world markets. This is the major conclusion of a report entitled *Competence and Competitions* prepared by the Institute of Manpower Studies for the National Economic Development Council (NEDC) and the Manpower Services Commission which compares, for the first time, British investment in training and human resources with that of West Germany, Japan and the United States. All three of these countries clearly recognize education and work competence as an important key to their economic success, some major companies in Japan and the United States, for example, investing between 2.5 per cent and 3 per cent of their sales revenue in training.

Young people in Germany, Japan and the United States are better equipped for working life than their British counterparts, the report says, because more of them participate in education and training programmes and for longer periods. In Japan, 84 per cent of school leavers voluntarily continue their education to the age of 18, in Germany the figure is 70 per cent, and in the United States 73 per cent of those aged between 16 and 24 are enrolled in some form of further education. It is quite sobering to realize that, in Britain, education beyond 16 is the exception rather

than the rule; two-thirds of all 16 year olds enter the labour market.

Britain trains only 15,000 graduate engineers a year compared with Japan's 70,000. But, the report says, the most acute shortage is among craftsmen, technicians and middle-level engineers, and Britain must counteract the decline in apprenticeship training by creating alternative training routes.

Education and training in Germany, Japan and the United States is funded primarily by industry. In Germany, for example, employers bear 80 per cent of the cost of the 1.67 million apprentices under training. All three countries have responded to the recession by accelerating their training programmes and so investing money in the future.

The report makes twenty-four specific recommendations. Among other things, it calls for measures to enable 80 per cent of young people in Britain to enter the job arena with qualifications relevant to their employers and for Britain to emulate Germany's achievement of having 12 per cent of its adult workforce on work-related education and training. Industry, it says, must take the responsibility for funding new engineering training facilities. The report calls on the government, trade unions and employers to make a positive commitment to these aims.

Marcus Chown

European high-energy physics

Poor marks for enterprise

IN the thirty years since the establishment of CERN, the European Organisation for Nuclear Research near Geneva, the laboratory has produced only two "crucial discoveries" compared with a dozen or more at United States laboratories, whose total cost is similar, and may even be less.

This is the bottom line of a lengthy balance sheet, with many other conclusions and observations, drawn up in nearly two years' work by social scientists Ben Martin and John Irvine, of the Science Policy Research Unit at the University of Sussex, and published today (6 September). The two-part report is certain to be influential in this particular reappraisal of its membership of CERN.

The first CERN discovery categorized as "crucial" was in 1973 ("neutral currents", a new form of weak interaction). By then, US laboratories had discovered a second neutrino, the omega-minus (the beginning of the quark model), charge-parity-violating interactions, deep inelastic scattering (which indicated that the proton really had components, now known to be quarks) and more. CERN, on the whole, has been thorough but dull, say Martin and Irvine — CERN lacked "bzzazz". But the second of CERN's crucial discoveries is very recent (the intermediate vector bosons W and Z found last year), is attributable to a new adventurousness at CERN and, according to the review, "to a certain extent the balance of power in experimental high-energy physics between North America and Western Europe seems to have reached a turning point".

Martin and Irvine are famed (or notorious) for their previous critical assessments of the Jodrell Bank radiotelescope, British astronomy (notably the Isaac Newton telescope) and the Daresbury electron synchrotron NINA. But their review of CERN is more important as it may affect real policy in two ways: first, a British scientific committee (see box) is studying whether Britain should remain a member of CERN, given the present £27-million price tag; and there are indications that Martin and Irvine's much-improved approach to scientific assessment in the CERN report is finding favour among British policy-makers, and could be applied to other fields. (A study has already been made of protein crystallography, for example, and delivered to Sir David Phillips, chairman of the Advisory Board for the Research Councils, which allocates basic research funds among the different research councils in Britain.)

But does the Irvine and Martin method work? The two researchers' earlier reviews drew criticism that in assessing scientific productivity (or the lack of it) in terms of indicators of publication volume, citation frequency and peer ranking of laboratories, they ended up with too bleak a

picture of what began to seem "targets" rather than mere subjects for research. There were mitigating circumstances, and forms of productivity unmeasured by the indicators.

But Martin and Irvine's present effort — two papers published in *Research Policy* — can hardly be criticized on those grounds. The CERN survey is sophisticated, and the two investigators clearly recognize a development in their technique. For example, they previously described their method as one of "converging" indicators, thus begging the question of whether or not the indicators do indeed converge on a single black-or-white assessment of a laboratory. In the case of CERN, the indicators clearly do not converge, and, write the researchers, "the results based on each indicator need to be carefully interpreted". Interpret they do, basing their analysis on 182 interviews with particle physicists from the Soviet Union to the United States.

High-energy physicists, even those at CERN, who have seen draft versions of the papers describe the analysis as fair, balanced and (mostly) accurate. CERN is admitted to have just missed the boat on many occasions. The two quarks called charm and bottom, which highlighted a revolution in physics in the 1970s described by Martin and Irvine as "the American Revolution", could have been found at CERN, the study claims. Missing the discovery of these particles was described by one physicist interviewed by Martin and Irvine as "a tremendous failure".

CERN even produced serious errors. One particle, the A_2 , was claimed — falsely, as it turned out — to be split, or

European dimension

SIR John Kendrew, the molecular biologist who is chairing a committee looking into Britain's future commitment to CERN, said last week that the Irvine and Martin report (see above) will be distributed to all the members of the committee — but along with many other papers it had received ("too many actually", said Sir John).

His committee will report "early next year" to the Science and Engineering Research Council, together with the Advisory Board for the Research Councils, and the committee plans to visit CERN in October, Sir John said.

Just what impact the committee's views will have remains to be seen — Sir Keith Joseph, Secretary of State for Education and Science, announced on setting up this committee that its scientific review would be "without prejudice" to a final (political) decision on CERN membership. The final decision will have to be taken in the wider context of Britain's whole relationship to Europe. Robert Walgate

twofold, creating a puzzle which absorbed theoreticians for nearly five years during the late 1960s. But similar errors were made in the United States (the "high- y " anomaly of Fermilab which caused equal consternation in the 1970s). Here the balance sheet is even.

In terms of positive results, CERN's first big machine, the 28 GeV proton synchrotron (PS), though on line before the equivalent alternating gradient synchrotron (AGS) at Brookhaven, was no-



Dr Herwig Schopper

where near as productive at the beginning. Physicists attribute this, above all, to lack of experience in the design of high-energy physics experiments in Europe. Then the intersecting storage rings (ISR), designed to collide PS protons with each other head on, was a brilliant technical success — but failed to produce revolutionary physics. (It helped in understanding the quark structure of nuclear particles, but could have seen and not missed charm and bottom.) Martin and Irvine suggest the machine was underexploited by CERN management. The 400 GeV super proton synchrotron (SPS) that followed came too late — more than four years after Fermilab in the United States had creamed the energy region — and had to rely on tidying-up (which, nevertheless, it did well, with resources that were considerably greater than Fermilab's).

The lesson, if there is a single one, is that Europe's laboratory was run too much by committee, whereas American laboratories owed more to individual flair. (Maurice Goldhaber at Brookhaven, R. R. Wilson at Fermilab and Pief Panofsky at Stanford Linear Accelerator Center are singled out.) But Martin and Irvine say that things are changing at CERN under director-general Herwig Schopper and that a more individualistic style has emerged.

Nevertheless, CERN has committed itself now to just one future machine and its 15-mile tunnel, the electron accelerator LEP now under construction. A future and more controversial report by Irvine and Martin will discuss the prospects for LEP, and at least one official involved in the British decision to support it has said he wishes he had had the report before him when he said yes.

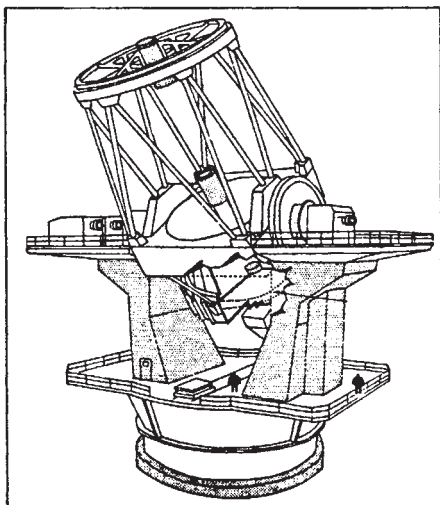
Robert Walgate

Japanese astronomy

Plan for telescope on Hawaii

JAPAN's astronomers, led by Professor Kei-ichi Kodaira of the Tokyo Astronomical Observatory, have announced plans to build what could be the world's largest optical telescope on Mauna Kea in Hawaii. A 7.5-metre single-mirror telescope is being called for — a considerable advance on the Mount Palomar 5-metre, or the 6-metre telescope now apparently operating at Zelenchukskaya in the Soviet Union.

Whether the Japanese telescope turns out to be the world's largest depends upon how US plans for a multiple-mirror



15-metre telescope (the NTT, new technology telescope), possibly on the same site, come along. Records, anyway, are not what the Japanese are after: the telescope is being designed to complement, not compete with, NTT and to mesh neatly with Japan's proven capability in millimetre-wave astronomy and X-ray satellite astronomy.

Although the telescope plan has only just been made public, it has been vigorously discussed for some years. Ten years ago, the astronomy committee of the Science Council of Japan — the major representative body for scientists through which virtually all plans for large-scale facilities must pass — recommended building a 4-metre telescope. In those days, as now, Japan's biggest optical instrument was the 1.88 metre installed in 1960 at Okayama.

The 4-metre telescope was not built, however, and much of the astronomical community's energy went instead into backing the construction of the world's largest millimetre-wave telescope at Nobeyama. By the time attention had turned back to the need for an optical telescope, technological advances had been made and a 4-metre telescope was clearly too small, given that it will be 1990 before it is in operation.

Over the years two kinds of obstacles had also surfaced. First, Japan lacks any kind of central astronomical institute. The

'Tokyo Astronomical Observatory', which has the major share of top-class facilities with seven observatories including Nobeyama, is actually a part of the University of Tokyo and does not have the authority to speak for all universities and astronomers, either to the government or to those in foreign countries from whom cooperation is necessary. Second, Japan has no history of large optical telescope building. Although there is no bar to foreign manufacturers, the newness of the design means it will be essential to have engineers close by to work with — and to charm with the technical challenge of the problems, rather than potential profits, for telescopes are not going to sell in bulk.

While neither of these problems has actually been solved, there is confidence that the Tokyo Astronomical Observatory can gradually move towards a central institute status similar to that held by ISAS and KEK (the space and high-energy physics research institutes) and that while international cooperation may be needed for the mirror design, Japanese engineers can handle the tracking and mounting.

The next question, which must be settled this year, is mirror design itself. There are advocates of both the honeycomb type pioneered at the Steward Observatory of the University of Arizona and a solid meniscus. Either way, the telescope aims at high performance not only in the optical region of the spectrum but also well into the infra red, with a resolving power around 0.1 arc seconds and a field of view wide enough (around 0.5 degrees) for efficient observation of 20th magnitude stars. This performance will complement that of NTT, which in aiming for a very large collecting area that will allow spectroscopy of extremely faint stars, will inevitably sacrifice some resolution (0.3 arc seconds has been cited), and the space telescope,

which will have no infra red facility (at least to begin with) nor the combination of high resolution and wide field of view that the Japanese telescope will be able to offer.

Prime scientific objectives for the new telescope will obviously be stellar and planetary formation and the formation and evolution of galaxies. As astronomy is getting 'redder and redder', the infra-red capacity will be of vital importance and allow a look back to early evolutionary stages of great cosmological significance. As two-dimensional infra-red detectors become available, the capacity of the telescope can be further expanded.

If the project gets the go-ahead, it will break political as well as scientific ground. At present, the Japanese Government owns no major research facilities abroad (other than Antarctic bases) and a move to Hawaii will be a major piece of 'internationalization' requiring legal changes and the passage of a couple of bills through the Diet. Contacts have already been made with the University of Hawaii and the state governor for there is not much space left on Mauna Kea: with a proposed limit of 13 telescopes, six have been built, two planned and more are expected to be listed soon.

The astronomical community is expecting that all the basic plans for the telescope will be complete in a few months. Beyond that it is up to the University of Tokyo, rather than the observatory, to submit the request officially to the Ministry of Education, Culture and Science and for it to press for funds — estimated at around 20,000 million yen — from the government. If all goes well, money for a two-year evaluation programme will be granted beginning in 1986 and will be followed by five years of construction.

There is no official word yet, but the astronomical community seems confident that the money will be found. Behind-the-scenes opinion sampling which always precedes the emergence of a decision in Japan appears to have been going well. By 1986 they should know for sure. **Alun Anderson**

Open University cuts

THE Open University (OU), in common with other British universities, is facing a cutback in government financial support. The proposed cut comes after four successive years in which OU has seen its state aid shrink in real terms. Meanwhile, its efforts to economize have resulted, over the same period, in an increase in the proportion of total university expenditure met by non-government sources from 15 to 25 per cent.

OU is concerned that the cuts will lead to a reduction in its student numbers and its courses and will also seriously limit its ability to provide teaching through television and radio programmes, and it disputes the government's assessment of the precise magnitude of the planned cut. Sir Keith Joseph, Secretary of State for

Education and Science, has insisted that the cut, over the three-year period until 1986, will amount to just £4.5 million. OU claims, however, that it will have to make a total reduction in expenditure of some £13.2 million by 1986, and has gone to some lengths to prove its point.

In arriving at its figure, OU has estimated the net increase in its costs (1983-86) due to inflation and 'unavoidable' costs (equipment renewals, superannuation increases, etc.), after taking into account the expected rise in income from student fees during the period.

About 100,000 students were enrolled at the Open University last year, Britain's only university with no formal entrance requirements and open to people who, through financial or job constraints, cannot attend a full-time institution.

Marcus Chown

Space shuttle

Discovery takes off at last

Washington

OFFICIALS of the National Aeronautical and Space Administration (NASA) could almost be heard breathing a collective sigh of relief last week when the temperamental shuttle orbiter *Discovery* finally cleared the launch tower at Kennedy Space Center, Florida, on 30 August. Two previous launch attempts were aborted with the crew already strapped in and ready to go, and a scheduled launch on 29 August was called off after deficiencies were found in computer software controlling critical flight functions. NASA has tried to keep its commercial customers happy by rescheduling satellite deployments, but at the back of everyone's mind is the knowledge that NASA will now be able to launch only 6 shuttle flights this year even if there are no further problems. Originally 10 were planned.

The 29 August attempt was abandoned because simulations confirmed that under certain emergency conditions (such as the failure of one of the five on-board general-purpose computers), commands to the shuttle's "major event controller" could be delayed by as much as one second, enough for them to be rejected. The controller switches such critical events as the separation of the shuttle's external fuel tank and solid-fuel booster rockets, not to mention the bolts that secure the orbiter to the launch pad. NASA stresses that the postponement was a "safety first" decision and that a launch on 29 August would probably have been successful.

The difficulty appears to have been caused ultimately by inadequate testing procedures, since NASA insists that the on-board computers and the event controller had consistently performed to expectation in pre-flight simulations. The timing problem was first noticed during separate tests on software under development for a future shuttle mission. Parallel tests were then run on *Discovery*'s computers and a similar effect confirmed: delays arose when the system had to deal with several events occurring almost simultaneously. The difficulty was rectified by a software patch, and go-ahead for the 30 August launch was given after a hastily-convened telephone conference of engineers. The inability to foresee exactly how a system of interacting computers will behave under non-standard conditions is presumably a problem that will not easily go away, because programs and hardware are continually being modified.

NASA was putting a brave face on the delay before last week's successful launch, and spokesmen were keen to point out that the last fault was only a potential one, and then only under worst-case conditions. Indeed, one suggested, it was to NASA's credit that it employed engineers able to raise such hypothetical problems. Whether

NASA's customers will be able to see further delays to the shuttle programme in quite this light remains to be seen. American Telephone and Telegraph (AT&T), whose Telstar 3 satellite was reassigned to the present mission after the original failure to launch *Discovery* in June, declares itself content with the service given by NASA so far. However, AT&T has not directly lost out as a result of NASA's latest rescheduling, which involved combining parts of the payloads of two separate missions. Less pleased, presumably, is Hughes Communications Services Inc., whose LEASAT-1 satellite has been held up by shuttle breakdowns, and a movie company whose cameras have had to be left behind.

Soviet space programme

Remote sensing in prospect

FURTHER Comecon participation in the Soviet manned space programme is not foreseen in the "near future", Academician Vladimir Kotel'nikov told a press conference in Moscow last month. "Very broad and successful" international cooperation of the socialist countries of the world would, however, continue, he said. The "most impressive", he noted, will be the planned Vega mission to be launched in December, in which a two-part probe will be sent to Venus, one part then making a soft-landing on the planet and the rest continuing the rendezvous with Halley's comet, passing through the tail (at some 10,000 km from the head) in March 1986. The 130-kg Venus payload will include equipment developed in Bulgaria, Hungary, Poland and Czechoslovakia, and also Austria, France and West Germany. East Germany will provide the ground equipment for decoding the signals.

Apart from Vega, future *Interkosmos* projects seem likely to concentrate on what has always been one of the main aims of the programme: the use of remote sensing to aid the economies of the member nations. Photography from space of the homeland of the "guest" cosmonaut always formed part of the manned *Interkosmos* flights (although in the case of the Cuban cosmonaut this was apparently carried out by his Soviet "hosts" after he had returned to Earth). An *Interkosmos* symposium in Dushanbe last November, in which the *Interkosmos* cosmonauts took part, summed up the experience of the manned programme with particular emphasis on the value of remote sensing for locating mineral deposition and "enhancing" agricultural productivity.

The annual conference of leaders of the national coordinating bodies of *Interkosmos*, which met in Prague shortly

Besides three commercial satellites, *Discovery* was carrying three major NASA experiments to provide information that will be needed for designing a space station. These employ a 30-metre long folding panel such as might be used for solar cells; on the present flight, however, most of the cells will be dummies. Besides characterizing the test cells' performance, the array will be used to gather information on structural vibrations. The shuttle is also carrying a continuous flow electrophoresis system belonging to McDonnell Douglas and Ortho Pharmaceuticals Inc., which will further examine the feasibility of commercial pharmaceutical manufacture in the zero-gravity environment of space. Earlier versions of this experiment flown in previous shuttle missions have given encouraging results, but the partners are not telling what exactly they have in mind to produce.

Tim Beardsley

before the Dushanbe conference, had likewise come out strongly in favour of remote sensing, the most practical route to making cooperation in space "more efficient".

A major step in implementing this joint remote sensing programme began last week in north-west Azerbaijan, involving representatives of all the European Comecon countries except Romania, working in conjunction with the three Soviet cosmonauts aboard Salyut-7. The ground team will make observations of the "spectral characteristics" of natural objects in the area (including the Mingechaur reservoir), which would be coordinated with aerial photographs and also photographs and spectrographs taken from the Salyut station. The orbital and airborne data are received by a special automated data-processing unit developed in Baku. The experiment is intended both to develop the "fundamentals of remote sensing of the environment" and also to provide practical recommendations for the utilization of agricultural land, pasture and reservoirs. For the Comecon participants, it is primarily a training exercise for future projects in which they will cooperate with Soviet space-crew passing over their homelands.

Interestingly, the experiment is coordinated not from Moscow or Baikonur, but by the Institute for the Remote Sensing of Natural Resources of the Azerbaijan Academy of Sciences. This institute has already obtained good results in predicting floods and mudslides in Azerbaijan, monitoring the state of the pastureland and forecasting the yield of the cotton crop. Delegation of coordination to the Academies of the Union Republics seems to be becoming a feature of the *Interkosmos* programme; the Vega mission is being coordinated by the Ukrainian Academy.

Vera Rich

Product contamination

FDA sends in the marshals

Washington

US MARSHALS acting for the Food and Drug Administration (FDA) last week seized \$4 million-worth of tissue culture and related products belonging to Flow Laboratories Inc., a major supplier to clinicians and researchers. The FDA action was taken following an inspection in April that found numerous instances of bacterial contamination of products and violations of FDA's code of good practice.

A federal judge later allowed Flow to recover raw materials and products used only for research. Flow attorneys argued that the FDA action was unlawful because marshals took "everything they could lay their hands on", and that cell lines would quickly die without attention. The company had been forced by the FDA action to close its manufacturing facility at McLean, Virginia.



Flow does not dispute most of the hundred or so complaints listed by FDA in April, but, according to Douglas Poretz, director of corporate affairs, the company has since made vigorous efforts to comply with the FDA observations. The complaints originally made by FDA allege that procedures for testing contamination were inadequate, that "clean" and "dirty" areas were not kept separate and that sterilization equipment failed to work to specification. Several products were not routinely tested in the manner claimed and significant contamination was present in others. In some instances, according to FDA, the company had failed to notify customers after a faulty batch was detected.

Flow says that after the April inspection, several senior staff were fired and outside consultants were brought in. But problems continued, and more than 10 product recalls were made during July. An anonymous Flow employee telephoned FDA to say that some products known to be contaminated were being shipped to customers. FDA finally acted on the basis of the product recalls without carrying out a second inspection.

Flow General Inc., the parent company,

has been through a disastrous 2 years and has sold off parts of its operation after sustaining losses last year of \$10 million on sales of \$129 million. Legal difficulties over a defence contract led to many staff resigning, and Flow Laboratories' factory in Scotland had to recall many products because of mycoplasma contamination from the water supply. A trial to examine the legality of the FDA action will be held on 12 September.

Tim Beardsley

French nuclear energy

Overpowered

FRENCH pressurized water reactors (PWRs) now provide more than half of French electric power, and last year "had the best performance ever" after a rather worrisome year in 1982, according to a spokeswoman for Electricité de France (EDF), the French national utility.

In 1982, French reactors were troubled by problems with the radiation embrittlement of certain clips ("broches") which held fuel in place, which resulted in long shutdowns for repairs and consequently very poor operating figures for that year. It was feared that the French method of large batch production of a very small number of designs might have been cheap, but risky. If there were a fundamental flaw in the design, at worst all the reactors could go down, and French electricity production would be halved. What had seemed like sense began to seem like foolhardiness.

But all along EDF claimed that these were mere teething troubles. The French nuclear system had grown so rapidly, most of it was very new: the *broches* problem was a problem of running-in, said EDF (crossing its collective fingers meanwhile).

Now, however, the *broches* problem is past and all is well. In the first half of 1984, French PWRs have turned in availabilities (energy produced divided by energy theoretically capable of production, if the reactors all ran full-time) of over 80 per cent, compared with figures around and under 60 per cent a year earlier. West Germany and Japan are said to be showing similar good results.

But the problem in France now is what to do with all that electricity. The French Government, in announcing a slimmed-down nuclear construction programme last year, asked EDF to aim at increasing French electricity consumption by 40 per cent by 1990, a task which requires finding some inventive new uses for electricity. Domestic electricity consumption has indeed risen this year by about one per cent a month — but there are no obvious reasons why (the French economy has been stagnant). The other possible opportunity of filling the gap is export. Given the tardiness of the other European power programmes in substituting for coal and oil, this must be the greatest opportunity facing the French utility in 1990.

Robert Walgate

French universities

Now one for Le Havre

THE new French minister of education, M. Jean-Pierre Chevènement, is conforming to his old, expansionist style, and belief in investment in "grey matter". He has created a new university.

This will be the Université du Havre, a university for the channel port of Le Havre, the third port of Europe, the twentieth city in France and one that has been seeking university status for its various colleges for some fifteen years.

But "the university of where?" asked a surprised spokesman for the Centre National de la Recherche Scientifique (CNRS), France's principal source of funds for basic research, when confronted with the news on Monday. Admittedly the university had been created during her holiday (last Tuesday in fact), but this may be a small indication that the university is not immediately going to startle the world.

Actually, Le Havre will be the 64th French university, counting the vast 13-university University of Paris as one, to compare with 48 in Britain (counting the University of London as one), for about equal populations in the two countries. The comparison reflects the effects of the troubles of May 1968, when student pressure and political expediency led to the creation of many new universities in France and elsewhere on mainland Europe, but few in Britain.

The expansion might have been thought to be over in France, but that was reckoning without M. Chevènement — and the fact that the new Prime Minister, Laurent Fabius, owes his seat in the national assembly to the electorate of the Seine-Maritime department, which includes Le Havre.

The university is expected to concentrate on matters concerning international trade, and will begin with a £3 million cash grant to create a 6,300 sq. metre campus. It will combine an existing IUT (an institut universitaire de technologie, something like a British polytechnic) of 1,000 students, and other units that have previously depended on the Université de Rouen, 40 miles up the Seine. Its creation thus runs counter to policies observable in other countries (notably Britain), which tend towards a combination of facilities among universities.

According to figures collected by UNESCO, the United Nations Educational, Scientific and Cultural Organisation, France ranks fifth in the world in terms of the proportion of the population studying at university after the United States, Sweden, Japan and the Soviet Union. West Germany and Britain follow after France.

Robert Walgate

Too little Indian zeal

SIR — To my mind, the basic problem with Indian academics is that their sense of social responsibility is often regrettably poor. Only a very few educated people are ready to produce an amount of work that will justify their salaries. Even in a research institution such as the Tata Institute of Fundamental Research, where the annual input is equivalent to US\$6 million, the annual output is about 270 research publications in the journals, shared by more than 300 top-level scientists. These scientists are potentially as capable as their counterparts in the West, but we become lazy in our home country. The very same people, when abroad, are compelled (possibly by example) to work hard, each producing as many as 5–10 papers a year. I am not saying that quantity is a measure of quality, but I certainly believe that, for the average research worker, quantity does beget quality.

The situation is much worse in Indian universities and other research organizations, which submit thousands of eye-washing project reports to their financiers, in preference to publication in one of the standard journals. Mainly on the basis of these obscure reports, however, the government measures national progress. Notwithstanding the rosininess of any such picture, the country seems to receive a poor return for its heavy investment. People are adequately paid for working, but the moral sense of their official responsibility fails to meet the minimum standard.

Mrs Indira Gandhi made a very brave attempt to improve matters by imposing emergency rule in 1976. All officials in positions of responsibility were forced to keep full office hours and to carry out their duties properly. This obviously irked all the educated parasites, who successfully brought about a ballot-paper revolution against her. So the poor lady has now learned that morality cannot be taught by force. We must rely on an appeal to people's individual consciences for any improvement — and I have written this letter in order to make such an appeal. A country can progress only if every citizen is willing to carry out his or her job responsibilities with sincerity and commitment. That is all that is needed in the first place, a step which has already proved to be the prime key to success in the West, the Soviet Union, China and Japan.

Many important plans in India have already failed because neither the planners nor the executors did their jobs properly. People are willing, for as long as a year if necessary, to weigh all the pros and cons before for example buying a motor car for themselves, but will lay out a hundred times as much on an expensive official project after as little as two weeks consideration. If that project is doomed to fail, it is not because of any failure of hands or brain, but because of a lack of commitment. The

chance of success depends much more on this factor than merely saying later that the project had been too ambitious. One must go for ambitious projects, but wholeheartedly.

Another problem for India is that it is producing more specialists than it needs. In the United Kingdom, only 2.5 per cent of students go to university, but in India, the fraction is about 15 per cent. This means not only that some incompetent students sneak into higher education, but also promotes a brain drain to other countries or to other types of lucrative jobs. The aim of the universities is quite lost in the burden of teaching, while the research institutions fail to hold the meritorious scholars, because of either poor rewards or poor guidance. The latter seems to be the greater problem. Unless the professor or director is prepared to do more brain work than his subordinates, there is no justification for either his rank or the wastage of national money on him. Only a very committed leader can create an atmosphere conducive to equal commitment from his staff. Will the right people ever set the right examples?

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Suggested remedies

SIR — For the sake of completeness of your appraisal of Indian universities (*Nature* 308, 581–600; 1984), I would like to add the following comments on higher education in India.

Since independence in 1947, India has taken big strides in higher education, especially in science and technology. In the past two decades, the number of institutes of higher learning has increased enormously, but the provision of new institutes without consolidating the existing ones has had an undesirable effect on the quality of education. Indeed, many institutes have become degree-granting bodies rather than places of learning.

Almost all universities suffer from the age-old problem of "inbreeding". Unlike those elsewhere, Indian students after graduation are usually employed at their own *alma mater*, and even a very bright graduate will have a tough time getting a job at any other university. As a result, some universities have certain departments specializing in only one discipline — that of the preceptor. This has essentially stifled the influx of new ideas and progress and also creates a situation in which the head of a department, being senior to the rest of the faculty, has an absolute say in the running of his department. His colleagues, most of whom were his own students, do not dare to voice their feelings. Since the fate of an

individual faculty member is decided by only one person, the spirit of competition is lost at the very beginning of tenure and every faculty member has to promote himself by means other than teaching and research. With the increasing linguistic chauvinism in the universities, science education has suffered a great deal. A developing country such as India needs ideas and literature from the advanced countries. But since education in English has been trimmed to promote one of the fourteen official languages of India, students cannot grasp the progress of developed nations. This is a self-defeating and shortsighted approach which has been forced on the country by self-promoting educationists and politicians. The effect has inevitably been an overall decline of science and technology education in India.

Striking has become a pastime for Indian students. Students go on strike for a difficult question paper, for failing grades or for compulsory promotion, but never for better teachers. Since the strikes are almost invariably resolved to the students' advantage, they have become an important tool in obtaining degrees, mainly because many students are admitted to university who are not sufficiently well qualified to be there.

India must find ways to eliminate the pitfalls inherent in the existing system. To start with, there should be a ban on appointments to a faculty from among its own graduates. English should be made essential for prospective scientists and technologists. There should be a nationwide qualifying test for university entrance. Educationalists and politicians should do everything they can to formulate progressive and farsighted policies on education and should rise above petty political advantage. It is after all education which eventually leads a nation.

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Red Sirius

SIR — The correspondence about Homer's wine-coloured sea reminds me about red Sirius. Several ancient authors call Sirius red, even though it is white with a slight bluish tint. This is another object which seems to have been called red even though blue would have been a better description.

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SIR — Homer's wine-dark sea is not the only such problem in Greek culture. It is well-known by astronomers that in Ptolemy's *Almagest*, Sirius is described as a red star, and there is no explanation on modern astrophysical grounds for such obvious disagreement.

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Molecular biology and cancer

A Nature conference next week is a modest celebration of the prospect that cancer will soon be understood. It does not imply that practical oncology will be transformed overnight.

THE question whether molecular biology has a contribution to make to the practice of medicine has now been answered. And positively. The half-dozen or so techniques for the diagnosis of genetic conditions and infection which are already in the hands of physicians are a sufficient proof of that. But when will molecular biology contribute something important to the practice of medicine, perhaps by helping to cure a disease and not by merely recognizing it?

For the past few years, there has been widespread excitement over the opportunities which seem to lie ahead for the treatment of genetic diseases and, more immediately, for the understanding if not the treatment of some of the many different forms of human cancer, which are in part at least genetic aberrations. That is one of the reasons why *Nature* is next week holding a conference on the subject in Boston, Massachusetts.

The opportunities in the field of cancer for molecular biologists are literally fascinating. They do cast a spell as can be told from the now wide currency of the word *oncogene*, which almost everybody knows of — and misuses. And there is a general opinion that oncogenes have something to do with viruses, whence the somewhat chilling suspicion that some forms of human cancer may yet turn out to be infectious (as, indeed, it seems that adult T-cell leukaemia is, at least in the coastal regions of southern Japan).

At times like these, it is therefore important to remember how long and tortuous is the history of the field that now seems so full of promise. It is well over half a century since the late Dr Peyton Rous, then a young man, first claimed that a sarcoma (in chickens) could be caused by a transmissible agent, an old-fashioned synonym for a virus. Rous persisted in the face of the sceptical politeness owed such a modest man and was rewarded, although not vindicated, by Richard Shope's discovery of the cell-transforming papilloma virus in the early 1930s. Only with development of techniques in the post-war decades was the reality of oncogenic viruses proved up to the hilt, first in rodents with polyoma (a DNA virus), then with the subset of the RNA viruses (collectively known as retroviruses) shown to cause cancer in animals.

More recently, the possibility of a connection between the retroviruses and the causation of human cancer has been answered with yes and no in an illuminating

way. The recognition within the past decade that there is often a close similarity between the nucleotide sequence of oncogenic retroviruses and certain unidentified genes in vertebrate cells was naturally a stimulus to investigation. Just over two years ago, three separate groups in the United States were able to show that a close similarity existed between the genetic structure of one strain of the ras retrovirus and a genetic structure in the DNA of cells taken from human bladder carcinomas.

By November of that year, two separate groups in the United States (at the Massachusetts Institute of Technology and at the National Institutes of Health) had been able to show that the aberrant structure, apparently that responsible for the causation of cancer, differed from that of a gene normally present in all human cells in apparently trivial ways. The normal version of the gene carries the code for a protein, called p21, whose existence had already been catalogued but whose function was unknown. The abnormal version of the protein, inferred from the structure of the abnormal gene, differs in only two amino acids in the sequence. From that point on, the attention of molecular biologists has been directed squarely at the human (and vertebrate) genome.

The picture that has emerged illustrates why the term oncogene is so commonly misused. At least in the case of the human bladder cancer (but probably many others), there is a normal gene which plays a crucial part in the biochemistry of all normal cells, and an aberrant version associated with the cancer. Strictly speaking, only the aberrant version deserves to be called an oncogene. In a careful world, the normal version would be called by a name indicative of its function.

So great has been the excitement since November 1982, that *Nature*, in a leading article on the outlook for 1983, offered the prospect that the causation of at least some forms of cancer would be understood within the year. In retrospect, that hope was over-optimistic; the function of p21 and its aberrant form have been glimpsed only in the past few weeks (see *Nature* 23 August, p.628, and references therein). But there have been other remarkable developments to make up for such disappointments as there may have been — the recognition that the oncogene called *myc* does appear to migrate from one chromosome to another in cases of human leukaemia and the startling recognition of a similarity of structure

of the gene responsible for the normal receptor for human epidermal growth factor and a true oncogene carried by the retrovirus known as erb-B. Whether the laboratory and clinical versions of the aberrant gene also interact with the biochemical system involving p21 remains to be determined, but it is hard to suppress the suspicion that the time is fast approaching when it will be worked out which properties of the cell surface have gone awry in cases of human malignancy.

These are only some of the reasons why the present is a time for modest celebration. But even now, it is far from self-evident that a better understanding of malignancy will help with the treatment of the human disease. That is a question that physicians rightly ask. They do so in the knowledge that even now, in other fields of human therapy, the development of successful drugs has ridden on the back of an understanding of how a disease is caused biochemically. It is only a hope that the field of cancer may be different, or that, say, the new techniques of molecular biology will provide a full understanding of the mechanism by which cells acquire genetic resistance to cytotoxic drugs.

The long history of people's attempts to deal with human cancer counsels caution. There have been too many crude disappointments for any other course to be safe. But even if the only certainty is that there will soon be a rounded understanding of the processes leading to overt disease, and ultimately perhaps of the interaction between genetic and environmental influences, it should at least be possible to put present strategies for the avoidance of cancer on a much sounder footing.

The role of *Nature* in such developments should not be misunderstood. Even a journal which enjoys the privilege of publishing many of the most important discoveries in such a field is not a scientific society in the strict sense but a vehicle for communication among active investigators and between them and the wider body of professional people. But the process of communication, in this field just as in any other, is more than the mere publication of information. The sense of excitement shared by those working in a promising field is also relevant. And there is much value in a forum in which those working at the bench can meet those whose work may be affected by what emerges. That is the spirit in which this conference has been designed.

John Maddox

Nitrogen cycle

Nitrate leaching from grassland

from R.E. White

RECENTLY, interest has revived in the effect that nitrate leached from agricultural land may have on the quality of surface and underground water used for human consumption. The consensus of scientific opinion, as presented in The Royal Society Study Group's report 'The nitrogen cycle of the United Kingdom', published in December 1983, is that most of the nitrate leached comes from arable land and represents approximately one-third of the nitrogen (N) applied annually to that land as fertilizer. The report concludes that little of the 900,000 tonnes of fertilizer N spread on grassland is leached out of the root zone. By contrast, results published in *Nature* this week (page 50), from J.C. Ryden, P.R. Ball and E.A. Garwood of the Grassland Research Institute at Hurley, suggest that loss of nitrate by leaching from intensively-managed grazed grassland may be five to six times higher than losses from comparable swards cut for hay or silage. The average annual loss from the grazed sward was 162 kg N per ha, which exceeded by far the range of 52–93 kg N per ha per year quoted in The Royal Society report for nitrate leached from arable land into the unsaturated zone of Chalk aquifers.

The Hurley researchers took soil and subsoil samples at 0.33 m intervals to a depth of 8 m from two adjacent plots (0.123 ha) on a loamy soil of the Frilsham series on Upper Chalk near Hurley. Both plots were sown to perennial ryegrass in the year April 1976 to March 1977 and fertilized with ammonium nitrate at the rate of 420 kg N per ha per year between April 1977 and December 1982. The 'cut' sward was mown every 4 weeks during the growing season while the 'grazed' sward was stocked with yearling steers for 1 week in 4 at an average stocking rate of 9.3 head per ha. From measurements of the drainage rate of nearby lysimeters, it was estimated that percolating water from the drainage season immediately following 1976–77 should have reached a depth of 5.5–5.8 m by December 1982. This coincided very closely with the depth of 6 m at which nitrate concentrations in the soil water below the cut and grazed swards were not significantly different. Above that depth, nitrate concentrations under the cut sward ranged from 5.4 to 13.3 mg N per litre, whereas the concentrations beneath the grazed sward exceeded the 'acceptable' range of 11.3–22.6 mg N per litre set by the World Health Organization.

Following the December 1982 sampling, the swards were ploughed and cropped to spring barley and winter wheat. A further sampling to 3 m depth in December 1983 confirmed the pronounced mineralization of soil organic N that occurs when grass-

land is ploughed. Allowing for the 80 kg per ha of fertilizer N applied in 1983, some 275–310 kg per ha of N in excess of crop requirements were mineralized, most of it being nitrified and therefore vulnerable to leaching.

Ryden and co-workers attribute the greater loss of N from the grazed sward to the inefficiency of ruminant animals in converting fertilizer N to protein, less than 20 per cent of the N input to grassland being recovered in animal products. The return of much of the ingested N in dung and urine creates 'hot spots' in the soil where the uptake of N by the grass fails to match the supply. The excess mineral N is then vulnerable to leaching.

The results of the new study are especially interesting because most previous work has been done on cut swards exposed to smaller amounts of N fertilizer (<250 kg

N per ha per year). The Royal Society report makes passing reference to nitrate concentrations of 10–100 mg N per litre in profiles beneath grass receiving >400 kg N per ha per year, but adheres to the central view that nitrate concentrations in water percolating below grassland are generally < 10 mg N per litre. Even so, much of the drainage water from less heavily-fertilized grassland will exceed the new European Community guide level of 5.7 mg N per litre. Grazing and fertilizer combined are likely to push the average concentrations above the new maximum level of 11.3 mg N per litre. As Ryden *et al.* point out, 37 per cent of the grassland of England and Wales now receives >200 kg N per ha per year and 5 per cent receives >400 kg N per ha per year. Further intensification of livestock farming is therefore likely to exacerbate the problem of nitrate pollution of water supplies at a time when politicians and the public are becoming more concerned about the potential health hazard of high nitrate in drinking water. □

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Astrophysics

Galaxies in collision

from M.G. Edmunds

GRAVITY never fails to surprise. The exotic predictions of general relativity have perhaps rather overshadowed the quite remarkable effects that can occur with nothing more than simple inverse square Newtonian force. Interesting things happen when galaxies collide, and recent dynamical simulations suggest that some quite strange morphological features of galaxies can be attributed simply to gravitational interaction.

The discovery of 'shells' — sharply defined extended arcs of low brightness — around elliptical galaxies was first reported in *Nature* four years ago (ref. 1 and see Fig. 1). The features were so unexpected that at first sight it seemed that they must be some kind of false diffraction image. Familiar artefacts of this type are the spikes seen on photographs around the images of bright stars, caused by diffraction at the structural supports of the telescope's prime focus. Nevertheless, investigations using different telescopes and of many galaxies have shown that the shells are real and occur around about 11 per cent of elliptical galaxies. No spiral galaxy has so far been found to have them.

The shells are generally aligned with the major axis of the galaxy, but do not completely surround it. Around a single galaxy there can be up to twenty shells, ranging over a factor of 30 in radius. Particularly intriguing is the observation that where several shells occur, they are generally interleaved, such that the next

shell out in radius from the centre is on the opposite side of the nucleus. The colours of the shells² are consistent with the colours of the population of stars in the galaxies, so it seems that the shells are composed of stars, though not particularly young ones.

How do stars come to be organized into such sharp-edged configurations? One possible explanation is that they formed there. Gas lost from stars in the main body of the galaxy³ or from an active galactic nucleus⁴ could blow out of the galaxy, cool and suddenly form into a narrow shell of stars as conditions became suitable. But why the regular spacing of multiple shells (unless it is the result of recurrent events) and, even more strange, why the alternation of shells on either side of the galaxy? An argument against the suggestion of an active nucleus is that the galaxies with shells seem, on average, to show radio emission less often than do similar galaxies without shells.

An explanation of shells on purely dynamical grounds⁵ was examined by P.J. Quinn (formerly of the Australian National University and now at Caltech) in his thesis work, and has now been published⁶. The basic idea is that a disc galaxy collides with a very much more massive — perhaps by a factor of 10 or 100 — elliptical galaxy. The massive elliptical acts as a potential well in which the stars from the disc become trapped. Computer simulations, performed with certain simplifying assumptions, show that the

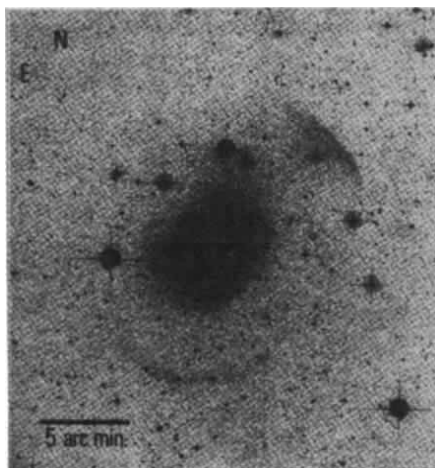


Fig. 1 The elliptical galaxy NGC 1344, showing sharp-edged shells which may be the result of gravitational interactions in a galactic collision (UK Schmidt plate, from ref. 1).

disc breaks up and the stars oscillate around in the potential. Provided the collision parameters are set correctly, and provided the original disc was dynamically 'cold' (did not have much random motion in the stars), then shell-like structures do occur. The number of stars per unit volume inside a galaxy is in general far too small for actual physical impact of the stars — they merely pass by each other and scatter off the gravitational potential of the system. The stars from the ruptured disc spend a lot of time at the ends of the radial motion of their orbits — rather as a simple harmonic oscillating pendulum spends most of its time at the ends of its swing — and the bunching of stellar orbits there is visible as a shell. The orbits evolve with time and more shells (essentially density waves) appear, interlaced on opposite sides of the centre, as is observed. The lifetime of a shell system, which ends when its structure becomes washed out by internal velocity dispersion, is predicted from these models to be some 2×10^9 years from the collision, during which time 10–30 shells would appear.

Astronomy still has a skeleton, not merely in its closet but actually knocking at the closet door, in the form of non-luminous mass in and around galaxies which we know must exist but cannot see. If correct, this gravitational-interaction picture could provide a most useful means of approaching the problem. Since the spacings of the time-evolving shells depend on the form of the gravitational potential, they can be used to probe the total (seen and unseen) mass distribution of the elliptical.

Despite their apparent success, Quinn's models face two major problems. The first is observational, the second theoretical. It might be expected that shell galaxies would occur where there was opportunity for plenty of collisions, but the reverse is observed: galaxies with shells are found preferentially in regions of low space density of galaxies. This objection can be weakened slightly by claiming that in

groups or clusters of galaxies, the velocities of collision would be too high for production of shells, or that subsequent collisions might destroy the shells.

The second, and probably more serious, problem is that shells are produced in the simulations only for a rather restricted type of collision. As Quinn is at pains to point out, other collision conditions lead to a veritable Modern Art gallery of structures. In a classic investigation some years ago⁷, Toomre and Toomre showed that the tidal gravitational interaction between two galaxies could lead to very peculiar structures — filaments, jets, arcs, bridges and tails — composed simply of stars pulled out in the collision. But in ellipticals, regular shell structure is found far more frequently than the complex structures that might be expected to result from arbitrary collision parameters. Undoubtedly some very strange structures do exist; sensitive photographic techniques and electronic light-sensitive arrays are beginning to reveal increasing numbers of jet-like structures in both spiral and elliptical galaxies. Just how many features can be accounted for on the basis of gravitational tidal interaction remains to be seen. Much more work can be done on dynamical simulations, although the almost unlimited range of masses, potentials, relative velocity vectors and internal dynamical states of the colliding galaxies makes this approach rather open-ended. Quinn's use of a fixed potential for the more massive galaxy must inevitably have suppressed collective effects — although it was sensible for computational convenience in an initial investigation.

The effects of collision on spiral galaxies have also been the focus of attention, with recent accurate mapping of the velocity field of a 'ring' galaxy in the direction of the Vela constellation by Taylor and Atherton⁸. Ring galaxies are fairly rare structures, characterized by a bright annulus, typically of young stars and associated glowing gas (Fig. 2). Two rival schemes have been put forward to explain their origins. Both explanations involve a normal spiral galaxy, with one scheme requiring collision with an intergalactic gas cloud, and the other collision with an elliptical (by contrast with the mechanism of shell formation, in the scheme for forming ring galaxies, the elliptical is much smaller than the disc galaxy). The critical ingredient is the gas, which is physically shocked and much of it forced over into gravitational collapse to form new stars.

The new velocity data, obtained with the novel 'Taurus' imaging Fabry-Perot system on the Anglo-Australian Telescope, are rather ambiguous. The velocity field is not what would be predicted in a head-on collision with an elliptical, that is, an expanding rotating ring. It may well be that the ring is actually folded back on itself, which could occur in collision with a gas cloud, as has previously been suspected in another peculiar galaxy, Arp 144. The

main difficulty, though, is that there are not enough intergalactic gas clouds to produce even the small number of ring galaxies known. Taylor and Atherton hedge their bets, and suggest both schemes may produce a share of the objects.

Colliding galaxies certainly seem to be coming back into vogue. Back in the 1950s, Baade and Minkowski proposed galactic collisions as the origin of strong radio sources. Although now it is massive black holes in galactic nuclei that are the preferred power source, it is becoming popular to attribute enhanced activity to the effects of collisions spilling fresh gaseous fuel down into the nuclei. The sudden 'starburst' of star formation that is induced by collision in the presence of a large amount of gas will in itself generate significant radio emission from new supernovae remnants. If there is a large amount of dust as well, absorption of visible and ultraviolet radiation and its re-emission at infrared wavelengths may give rise to the very strong infrared luminosities (up to 100 times the optical output) detected by the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Fig. 2 The Cartwheel galaxy, a giant ring galaxy, which may have been formed by collision. (Photograph by Photolabs, ROE, from an original negative by the UK 1.2 m Schmidt telescope.)

IRAS satellite in several apparently interacting systems. Infrared excesses from interacting pairs, seen in ground-based studies⁹, suggest that only one of the pair undergoes a sudden starburst.

A new catalogue¹⁰ of elliptical galaxies with shells has been prepared by the original discoverers, D.F. Malin and D. Carter, and contains over 130 candidates for further study. What an exciting prospect, given the rich diversity of complicated structures that can be generated by a low-velocity galactic collision. □

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MICRO-84

Electron microscopy 50 years on

from Colin J. Humphreys

IN 1934, *Nature* published the first electron micrographs of biological material. Fifty years later, the Royal Microscopical Society, in association with *Nature*, organized MICRO-84, an international microscopy conference and exhibition held in London from 9 to 13 July 1984. The conference was attended by about 800 scientists from all over the world.

One of the highlights of the meeting was a description by Albert Crewe (University of Chicago) of a revolutionary 0.6 Å point resolution scanning transmission electron microscope he has recently started to build. This represents a dramatic advance in resolution over existing electron microscopes, the best of which have a point resolution of about 1.7 Å. Modern electron microscopes have computer control added on, as it were. Crewe proposes to reverse this procedure and build his microscope around a computer system large enough to operate and control the microscope, and process and store the images. His computer system, comprising four computers with 3.6 Giga bytes of storage, has been donated by IBM, which demonstrates the importance that major microelectronics companies attach to high-resolution electron microscopy. If successfully built, Crewe's microscope should have major applications in biology, chemistry, materials science and physics.

Many microelectronic circuits are now so small that they cannot be resolved using an optical microscope, and an electron microscope is necessary simply to see them. D. Cherns (University of Bristol) and W.M. Stobbs and D.J. Smith (University of Cambridge) demonstrated how high-resolution electron microscopy can be used to determine the positions of atoms lying in and near the interfaces of semiconductor devices, so that their electrical properties can be understood.

In recent years, not only the resolution but also the analytical capabilities of electron microscopes have dramatically improved. It is now possible to analyse chemically very small regions of materials using an electron beam less than 10 Å in diameter, by detecting the signals (for example, characteristic X rays) generated when the incident electron beam is inelastically scattered by the specimen. Moreover, it is possible to measure the energy spectrum of the transmitted electrons by a technique called electron energy loss spectroscopy, which is sensitive to all elements and is increasingly being used for the analysis of both biological and non-biological materials.

If the fine structure of an electron energy spectrum is studied, a technique known as electron loss near edge structure (ELNES),

results can be obtained equivalent to those achieved on a synchrotron source, but for any atomic number element and with far higher spatial resolution (C. Colliex, University of Paris). ELNES tells us not only what atoms are present in a sample, but also their environment. Mick Brown (University of Cambridge) described some novel applications of electron energy loss spectroscopy which exploit the very high spatial resolution available, for example, the measurement of energy band gaps in insulators.

Topics covered in the wide-ranging programme included microsurgery, for-

ensic science, archaeology and art. Among the relatively new fields, C.F. Quate (Stanford University) gave an outstanding review of the advances in acoustic microscopy from a resolution of 10 µm 10 years ago to one of 300 Å, using helium, today, and G. Schmahl (University of Göttingen) described the latest results from his 500 Å resolution X-ray microscope, which is probably the best in the world. X-ray microscopy offers the prospect of studying living biological material with a resolution far better than that of optical microscopy, and with much less damage than is produced in an electron microscope. To be really useful, however, its resolution will have to be improved still further. Perhaps this will be achieved by the next meeting of MICRO, in 2 years time. □

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Vaccine development

Recombinant vaccinia for prevention of hepatitis B

from John Beale

THE idea of using vaccinia virus as a means of carrying other immunogens for the purpose of vaccination has seemed very attractive since Paoletti and Moss and their colleagues started to investigate the use of vaccinia recombinants in 1982 (Panicali, D. & Paoletti, E. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4927; 1982 and Mackett, M., Smith, G.L. & Moss, B. *op. cit.* **79**, 7415; 1982). The rationale is that the live virus will express a foreign gene inserted into its DNA when it is applied to an animal, which will subsequently produce an immunological response to the foreign gene product. So far, recombinant DNA technology has been used successfully to express various antigens in vaccinia, including hepatitis B virus surface antigen (HBsAg), malaria sporozoite antigen, and influenza and herpes glycoproteins, and the list is growing all the time. On page 67 of this week's *Nature*, Moss and his colleagues take the hepatitis story a stage closer to a practical vaccine by showing that chimpanzees can be protected against hepatitis by vaccination with live recombinant vaccinia virus.

Last year, Smith, Mackett and Moss reported the construction of a fusion gene between DNA encoding HBsAg and an early vaccinia promoter (*UCLA Symp. molec. cell. Biol.*, New Ser. **8**, 543; 1983). By inserting the fusion product into the thymidine kinase-encoding portion of the vaccinia genome such that thymidine kinase activity was no longer expressed, they could select the new recombinant virus by growth in the presence of 5-bromodeoxyuridine. The plaques were tested for HBsAg expression by dot-blot

hybridization and for HBsAg gene expression by immunological methods.

What Moss and his colleagues have now done is to vaccinate two chimpanzees with the HBsAg-containing recombinant vaccinia virus and one chimpanzee with wild-type normal vaccinia virus, and then challenge them with living hepatitis B virus. They find that treatment with HBsAg-vaccinia hybrids produces smaller lesions and lower antibody titres to vaccinia than does treatment with vaccinia alone. None of the animals produced antibodies to HBsAg after vaccination. On challenge with hepatitis B virus, however, the chimpanzee given vaccinia alone developed typical signs of hepatitis B virus infection: hepatitis surface antigenaemia, antibodies to the core antigen as well as to the surface antigen of hepatitis B (the production of core antibodies indicates some hepatitis B virus replication had occurred in the primed animal) and biochemical evidence of hepatitis. By contrast, the two HBsAg-vaccinia-treated chimpanzees, although producing no antibodies before challenge, were protected against the challenge, as shown by the lack of biochemical evidence of liver damage and of antigenaemia, and the rapid onset of antibodies to the surface antigen. They had thus been immunologically primed by the expression of HBsAg when the recombinant vaccinia was growing in the skin.

These are impressive results that pose in a sharper form the policy implications of this approach. The attraction is that the accepted and proven instrument of smallpox eradication can be adapted to the formidable task of hepatitis B prevention.

Indeed, as a result of the success of the World Health Organisation (WHO) smallpox eradication campaign, vaccinia has had magical powers conferred on it, not only by the populations that have benefitted; but also by some public health officials.

At the time of the first publication by Smith, Mackett and Moss, two problems were foreseen: immunity to vaccinia and the question of safety. These two issues remain. On any rational consideration of cost versus benefit, vaccinia should be acceptable in Third World countries for the prevention of hepatitis B. Such decisions are not always made rationally, however, and the use of a vaccinia hybrid for the control of hepatitis B in the USA, for example, would probably be more convincing to governments in Third World countries than hours of debate. For western countries, however, there is no incentive to use vaccinia with its known hazards, which caused it to be abandoned for smallpox control around the time that the WHO started its smallpox eradication scheme. Moss *et al.*'s finding that the lesions in the chimpanzees were less for the recombinant than for the wild vaccinia is encouraging, although more work is clearly needed. The problems of effectiveness and of interference from vaccinia antibodies during booster doses remain to be overcome.

As large amounts of hepatitis B surface antigens become available using recombinant DNA techniques of expression in yeasts and in mammalian cells, there may be no need to use a vaccinia carrier. Instead, it may be possible to combine hepatitis B vaccine with other non-living childhood vaccines, and to give the combination at birth or soon thereafter.

Priming of the immune response so that immunological memory is induced is one aim of a vaccination programme. Recently, Salk *et al.* (*Ann. clin. Res.* 14, 204; 1982) have argued forcibly that this would justify consideration of a single dose of killed poliovaccine for poliomyelitis control in tropical areas. The problem is the uncertain duration of memory in the absence of an adequate primary stimulus to produce antibody of both IgM and IgG classes. Further experimental data should soon be available to answer these outstanding questions for poliovaccine.

As to future research into the use of vaccinia as a carrier of immunogens, increased expression of the inserted DNA so that larger quantities of immunogen are produced is an important goal. Certainly, there is sufficient evidence to warrant continued research and the cautious extension of experiments in man. It is, however, much too early to think this promising line of research is a panacea for all the vaccine problems in developing countries. □

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Mathematical modelling

The cubic map in theory and practice

from Robert M. May

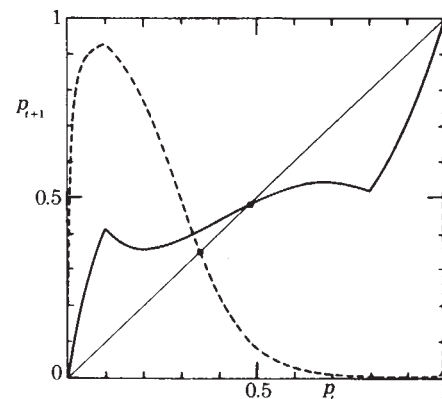
FOR many populations of temperate-zone insects, the adults emerge, mate, reproduce and die over a brief interval in the summer. The dynamical behaviour of such a population, with discrete nonoverlapping generations, may be described by a first-order difference equation of the form $x_{t+1} = f(x_t)$; the population density in generation $t + 1$, x_{t+1} , is given as some function, f , of the density in generation t , x_t . If this equation were linear ($f = \lambda x$), the population would simply increase (or decrease if $\lambda < 1$) in familiar exponential fashion. More commonly, however, the population will tend to increase when at low densities and to crash at high densities, corresponding to some non-linear function or 'map' with a hump (of which the quadratic $f = \lambda x(1-x)$ is the mathematically simplest example). It is now widely appreciated that such non-linear first-order difference equations with a single hump or critical point can unfold an astonishing richness of dynamical behaviour: as nonlinearities become more severe (specifically, as λ increases in the quadratic example), the original stable point gives way to a cascade of period-doubling bifurcations, successively producing cycles of periods 2, 4, 8, 16, ..., 2^n ; eventually an apparently 'chaotic' regime is entered, in which the trajectories generated by this deterministic process can be effectively indistinguishable from random noise^{1,2}. Many features of this bifurcation phenomenon are reproduced in a self-similar manner, on even finer scales, in successive period doublings, in ways that are generic to essentially all maps with one critical point^{1,3}. This subject, which first came to prominence in the early 1970s in studies of biological populations^{4,5}, has grown dramatically as more possible applications are found in the physical and engineering sciences.

What are the properties of maps with two humps? The canonical example of a map with two critical points is the symmetrical cubic $x_{t+1} = \lambda x_t + (1-\lambda)x_t^3$ (which is chosen such that $+1$ maps to $+1$, 0 to 0 and -1 to -1). A hint that the dynamics of the cubic are likely to be messier than those of the quadratic is provided by a theorem saying that maps with n critical points can have up to n distinct stable orbits for any given set of parameter values⁶. For maps with one hump, this means at most one stable state or attractor; for generically cubic maps, however, there can be two alternative attractors. Several authors have set out how two such alternative stable orbits can arise for the cubic map^{7,9}: when the originally stable point at

$x = 0$ becomes unstable (for $\lambda > 2$), it first bifurcates to give a single symmetrical cycle with period 2; as the map becomes more significantly nonlinear ($\lambda > 3$), this cycle does not bifurcate to give a 4-cycle, but rather splits to give two distinctly different (and unsymmetrical) 2-point cycles. Each of the separate 2-point cycles then unfolds the familiar cascade of period doublings to cycles with periods 4, 8, 16, ..., 2^n as λ continues to increase. These cascades display the self-similar properties emphasized by Feigenbaum³ for the quadratic map. For yet larger values of λ we enter the apparently 'chaotic' regime, where, again, there are two separate attractors for most values of λ . A detailed catalogue of all the possible cycles that lurk as a kind of kaleidoscopic fine structure within the apparently chaotic regime is much more complicated than for the quadratic map^{1,2}, though an explicit such catalogue has been given for all cycles with periods up to 10, in their order of appearance⁹. Methods for computing the total number of distinct cycles with period k have also been given⁷.

The motive behind the first studies of the cubic map was simply that it represented the next logical step beyond the quadratic⁷. Since then, however, the cubic map has come to people's attention in a variety of practical contexts.

Holmes¹⁰ studied equations that arise as the simplest description of a buckled beam that is subjected to forced lateral vibrations. This led him naturally to consider the cubic equation given above, as being the simplest dynamical relationship satisfying the symmetry requirements of his problem. Holmes then established that, for a wide



Relationship between the frequency of allele A in successive generations, p_{t+1} to p_t , for a system in which the fitness of host genotypes are determined by associations with pathogens. It is the general propensity to increase at low values and decrease at high values that is the characteristic of the cubic map. (From ref. 16.)

range of parameter values, "two distinct non-periodic attractors exist". His numerical studies of the more complicated buckled beam problem — using both a digital and an analogue computer — corroborated that, indeed, two distinct attracting states exist.

Perez, Jeffries and Testa¹¹ have experimented with an electronic circuit consisting of a harmonically driven p - n junction. They find this system to be characterized by period doublings and chaos of a kind that cannot be modelled by a map with one critical point. Guided by the numerical studies of Testa and Held⁸, they believe the symmetric cubic map may describe the essentials of their observations. Perez *et al.* also discuss similar dynamical phenomena, again possibly corresponding to a cubic map, arising in the Belousov-Zhabotinsky chemical reaction.

More generally, Pikovsky¹² has observed that the dynamical properties exhibited by the cubic map may help explain the kinds of transitions to chaos that are observed in systems with a particular symmetry: an example could be the transition to turbulence in a hydrodynamic system with symmetrical boundary conditions. An independent and more explicit example of this sort is provided by Coste and Peyraud's¹³ study of the Poincaré map generated by a model for Rayleigh-Bénard convection. For this symmetrical system, these authors find a bifurcation sequence of the kind described for the cubic equation above (and elsewhere they give an independent analysis of the bifurcation structure of the cubic map¹⁴).

To my mind, the most interesting application of the cubic map and its friends and relatives with two critical points lies in population genetics. In many situations, it can be that rarer genotypes — and hence rarer alleles — enjoy a selective advantage in the natural world. When this happens, the frequency, p , of the allele A at a diallelic locus will tend to increase when it is rare, and to decrease when the allele is common; that is, the map expressing p_{t+1} as a function of p , will tend to have two humps. If the frequency-dependent effects are exactly symmetrical, we will have the symmetrical cubic map discussed above, and more generally we will have some unsymmetrical map with two critical points. The ensuing frequency of the allele A may settle to a stable constant value ('balanced polymorphism'), or it may oscillate (in a unique cycle, or in one of two alternative cycles), or we may have apparently chaotic dynamics.

Hamilton¹⁵ has observed that the selective forces exerted by pathogens on their host genotypes are likely to be of just this frequency-dependent kind, with rarer genotypes being at an advantage because their particular diseases are less effectively transmitted by virtue of their low host densities. Hence host-parasite evolution may produce cyclic or chaotic variation in the prevailing host genotype from generation

to generation. Hamilton goes further to observe that sexually recombining host populations will undergo less severe oscillations or chaos than corresponding asexual ones, essentially because the existence of heterozygotes and of crossing-over in multilocus situations endows the sexual system with greater dynamical inertia than the asexual one. It can then be that, under strong frequency-dependent selection of the kind possibly associated with pathogens, sexual systems enjoy an average fitness more than twice that of the corresponding asexual system, due to this dynamical mechanism. (The detailed argument is complex, but I believe the basic mechanism is as set out here.) Hamilton's calculations use plausible phenomenological expressions for the frequency-dependence in host fitness produced by pathogens; later studies employing epidemiologically-derived fitness functions find that sexual systems can overcome their intrinsic twofold disadvantage only in the most exceptional circumstances, which tends to undercut the theoretical basis for the suggestion that parasites are responsible for the evolution and maintenance of sex¹⁶. The idea, however, remains a fascinating one, and it will ultimately stand

or fall on the basis of empirical tests of its predictions, several of which are underway¹⁷⁻¹⁹. Courting couples may feel that sex and the cubic map have little in common, but the fact remains that these two topics are intimately linked in current research on population genetics. □

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Atmospheric chemistry

Ozone increases in ground-level European air

from S.A. Penkett

EPISODES of photochemically generated ozone are now a common form of acute air pollution in Europe, whilst smoke fogs, which were typical of winter in London and other cities in the 1950s, have largely disappeared. The reasons are complex and are concerned with the changing emission patterns of primary pollutants and with the chemical changes that occur in the atmosphere. High ozone concentrations caused by photochemical pollution were first detected in Los Angeles in the 1940s. It was not until more than twenty years later that the first indications were found of a similar problem in Europe. Measurements of ground-level ozone then commenced, in Holland and at Harwell in southern England. These have produced clear evidence of the production of large amounts of ozone at ground level under anticyclonic conditions.

It is unfortunate that ozone measurements in western Europe started only recently, because it is difficult to detect a trend. By contrast, systematic measurements have been made at both urban and rural sites in the German Democratic Republic (GDR) since 1952. The results show a dramatic increase in the yearly average ozone concentration with the largest increase being observed at rural sites. Data from five sites located through-

out the GDR were published in 1979 but attracted little attention at the time (Warmbt, W. *Z. Met.* **29**, no.1, 24; 1979). Recent interest in ozone as a possible cause of damage to forests has highlighted the importance of this unique data set.

The measurements were made using the classical technique of iodometry in solution, which is subject to interference from other atmospheric gases. Modern methods of ozone measurement utilize an absolute optical absorption method to avoid these problems. The same technique was used consistently in the GDR measurements, however, and the imperfections were recognized. Interference from sulphur dioxide was particularly bad at urban sites until a chemical filter was introduced in 1972. At rural sites the interference was less and at one site, Cape Arkona on the Baltic Coast, it was totally absent.

The average ground-level ozone concentration at Cape Arkona showed an increase in the yearly mean from ~ 15 parts per 10⁹ by volume (p.p.b.v.) in 1956 to ~ 24 p.p.b.v. in 1977, the most recent year for which data are reported. The mean summer ozone concentration almost doubled, from ~ 18 p.p.b.v. to ~ 32 p.p.b.v. over the 21 year period. The maximum levels observed in photochemi-

cal episodes were considerably greater, reaching values of over 100 p.p.b.v.

There are no other ground-level observations of ozone in Europe going back so far. Nevertheless, data collected on the Hohenpeissenberg in southern FRG from 1971 to 1976 show a similar trend to those from the nearest GDR station over the same period. Probably the most numerous data for Western Europe have been collected in Holland. These measurements started in 1969 but are mostly urban and will be affected by large emissions of nitric oxide in the immediate vicinity. This gas reacts rapidly with ozone with the result that levels in urban areas tend to be lower than those in rural areas. The Dutch data show no overall increase during the 1970s.

The continuous upward trend in ground-level ozone concentration, seen particularly at rural sites, is unlikely to be a result of increased transfer of ozone from the stratosphere, which provides much of the ozone in the troposphere, but rather of a change in the efficiency of production *in situ* in the boundary layer. Photochemical production of ozone in the lower atmosphere occurs through a complex chain reaction involving free radicals. The essential precursors are oxides of nitrogen and hydrocarbons; emissions of both these species in Europe have increased substantially over the period covered by the ozone data. If Britain is typical, nitrogen oxide emissions have increased by about 50 per cent and emissions of hydrocarbons have more than doubled. Motor vehicles are the largest source of the chemically reactive hydrocarbons that are the most effective in producing ozone, and between 1953 and 1975 fuel usage in Britain increased about threefold. Most of the increase occurred before 1970, during a time when no systematic measurements were made either of the primary pollutants or of their effects on the ozone concentration. This emphasizes the unique nature of the GDR data.

The observed increase in ozone is indicative of an increase in chemical reactivity in the atmosphere which will probably be reflected in an increased rate of acid production. Indeed, amounts of other potentially acid species may be correlated with ozone. For example, the nitrate content of atmospheric aerosol and of rainwater in England has increased roughly by a factor of three since the mid 1950s. The nitrate aerosol and ozone trends both show peaks and troughs which are associated with meteorological factors, in particular with the amount of sunlight. There is, in fact, some correlation between high oxidant levels and good vintage years, particularly for the Rhine and Mosel wines grown at a similar latitude to the stations where the data were collected. Clearly, there is a strong case for sustained research into the nature of all chemical changes in the atmosphere that lead to the formation of strong oxidants and free acidity. These two chemical properties are strongly

linked, as the German word for oxygen, *sauerstoff* (literally 'acid stuff'), shows.

The known aspects of ozone chemistry in the troposphere have recently been discussed in the Swedish journal, *Ambio* (13, no.2, 61; 1984). The phytotoxic properties of ozone are emphasized, particularly in the context of forest damage in central Europe. The influence on climate of an increase in ozone concentration throughout the Northern Hemisphere is also considered, as it is possible that emissions of oxides of nitrogen and carbon compounds, such as carbon monoxide and hydrocarbons, from a variety of sources now affect the distribution of tropospheric ozone on a hemispheric scale. Thus, data from the

global ozonesonde network show, for the period 1968–1979, an annual increase in ozone concentration of 1 per cent in the height range 2–8 km at mid latitudes in the Northern Hemisphere. Ozone behaves as a greenhouse gas, that is, it absorbs infrared radiation from the Earth's surface and thus acts as a thermal blanket. Calculations suggest that a doubling in the tropospheric ozone concentration will increase the global surface temperature by approximately 1°C; this is of a similar magnitude to the value of 3°C calculated for a doubling in the CO₂ concentration. □

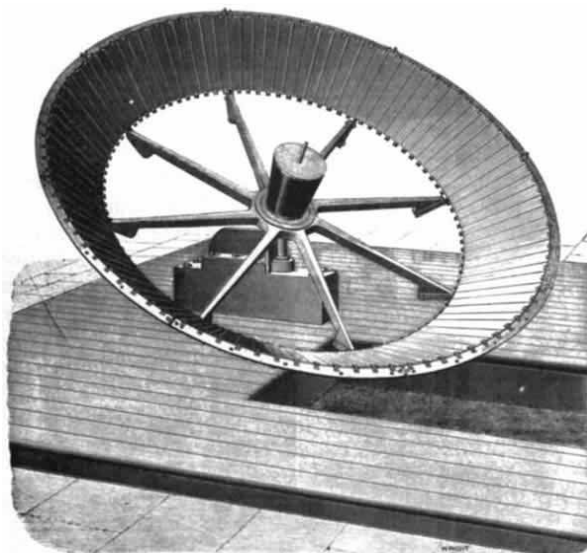
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100 years ago

THE TEMPERATURE OF THE SOLAR SURFACE

THE power developed by the sun motor, recorded in *Nature*, has established relations between diffusion and energy of solar radiation which prove that the temperature of the surface of the sun is extremely high. I have, therefore, during the summer solstice of 1884, carried out an experimental investigation for the purpose of demonstrating the temperature of the solar surface corresponding with the temperature transmitted to the sun motor. I have accordingly constructed an instrument of large dimensions, a polygonal reflector (see Fig. 1), composed of a series of inclined mirrors, and provided with a central heater of conical form, acted upon by the reflected radiation in such a manner that each point of its surface receives an equal amount of radiant heat in a given time. The said reflector is contained within two regular polygonal planes twelve inches apart, each having ninety-six sides, the perimeter of the upper plane corresponding with a circle of eight feet diameter, that of the lower plane being six feet.



Captain Ericsson's Solar Pyrometer, erected at New York, 1884.

The corresponding sides of these planes are connected by flat taper mirrors composed of thin glass silvered on the outside. When the reflector faces the sun at right angles, each mirror interprets a pencil of rays of 32.61 square inches section, hence the entire reflecting surface receives the radiant heat of an annular sunbeam of $32.61 \times 96 = 3130$ square inches section. The reflector and conical heater are sustained by a flat hub and eight radial spokes bent upwards towards the ends at an angle of 45°. The heater is composed of rolled plate iron, and provided with head and bottom formed of non-conducting material. A short tube passes through the upper head of the heater, through which a thermometer is inserted for measuring the internal temperature. The stem being somewhat less than the bore of the tube, a small opening is formed by which the necessary equilibrium of pressure will be established with the external atmosphere. It should be mentioned that the indications of the thermometer during the experiment have been remarkably prompt.

The result of the experimental investigation carried out during the summer solstice of 1884 may be thus briefly stated. The diffusion of the solar rays acting on the 20-inch heater being in the ratio of 1 to 10,241, the temperature of the solar surface cannot be less than $298^{\circ}.87 \times 10,241 = 3,060,727^{\circ}$ F. This underrated computation must be accepted unless it can be shown that the temperature produced by radiant

heat is not inversely as the diffusion of the rays. Physicists who question the existence of such high solar temperature should bear in mind that in consequence of the great attraction of the solar mass, hydrogen on the sun's surface raised to a temperature of 4000°C., will be nearly twice as heavy as hydrogen on the surface of the earth at ordinary atmospheric temperatures; and that, owing to the immense depth of the solar atmosphere, its density would be so enormous at the stated low temperature that the observed rapid movements within the solar envelope could not possibly take place. These facts warrant the conclusion that the high temperature established by our investigation is requisite to prevent undue density of the solar atmosphere. J. ERICSSON

From *Nature* 30, 465 (1884).

Polymer science

Novel helical conformation in a nylon

from Edward Atkins

X-RAY structure determinations undertaken so far on different nylons have shown that the chains crystallize in an extended planar zig-zag conformation. In the crystals, the vital amide groups (-NH-CO-) participate in interchain hydrogen-bonding in the plane of the zig-zag, generating hydrogen-bonded sheets which stack to form the three-dimensional crystalline structure. The properties and behaviour of the nylons examined until now all relate to this type of molecular architecture. In this issue of *Nature* (page 53), Fernández-Santín, Aymami, Rodríguez-Galán, Muñoz-Guerra and Subirana describe a novel structure for a nylon derivative, poly(α -isobutyl-L-aspartate): a contracted spring-like helix, stabilized by intrachain hydrogen bonds, which exhibits a number of similarities with the well-known α -helix found in proteins and polypeptides (see Fig. 3 on page 53). This novel spring-like conformation is of interest not only for its own sake and because of the implications it has for the properties of a nylon, but also because it highlights the link nylons can provide between synthetic polymers and proteins.

The nylons or polyamides represent a spectrum of synthetic polymers in which amide groups introduce a hydrogen-bonding capability into the structure. At one extreme, the amide groups occur very infrequently, and the nylons approach the conformation, behaviour and properties of polyethylene: an extended planar zig-zag chain associating with neighbouring chains through relatively weak van der Waals' interactions. Near the other end of the spectrum, where the amide groups are separated only by one or a few CH_2 units, is the structure, known as nylon-2, which is chemically identical to polyglycine — a polypeptide or simple protein — in which two carbon atoms ($\text{-CH}_2\text{-CO-}$) occur between successive nitrogen atoms in the chain. (The ultimate polyamide is nylon-1.) The bulk of the nylons used industrially have amide groups distributed with a frequency intermediate between these two extremes. A common polyamide is nylon-6 with six carbon atoms between successive nitrogens. It might be predicted, *a priori*, that as the frequency of amide groups increases and the nylons take on a similar chemistry to that of polypeptides and proteins, they would be capable of mimicking their biopolymeric counterparts.

In proteins, only a relatively small number of regular conformations have been found, of which the α -helix, the 3-fold or collagen-like helix and the β -sheet are the most prominent. These regular

structures are ubiquitous in fibrous proteins (Fraser, R.D.B. & MacRae, T.P. *Conformation in Fibrous Proteins*, Academic, New York; 1973) and occur as domains, connected by less regular segments, in the convoluted conformations found in globular proteins, for example myoglobin, haemoglobin and the enzymes. β -sheets are extended protein chains, hydrogen-bonded together to form sheets. They occur in many insect silks, and can be thought of as the biopolymeric analogues to the traditional nylon struc-

tures. Unlike the β -structures, which are stabilized by interchain hydrogen-bonding, the α -helix is a contracted spring-like helix stabilized by intrachain hydrogen bonds, and is found in many keratinous tissues such as hair.

The novel structure discovered by Fernández-Santín *et al.* for nylon-3, which closely approaches the frequency of amide groups in proteins (variants of nylon-2 backbone), is a departure from the traditional nylon structures and represents the first of what is hoped to be a family of self-stabilized spring-like helices for nylons. These substances should exhibit behaviour and properties quite different from those of previous nylons, but similar to those of the extensively studied α -polypeptides and α -proteins. □

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Anaesthesia

Glycine receptor protein the solution?

from W. Graham Richards

ALCOHOL added to a fish tank containing newts will anaesthetize the newts and they will keel over unconscious. Surprisingly, a subsequent increase of the hydrostatic pressure on the water of the tank reverses this anaesthesia and the unconscious creatures revive and will swim normally under the increased pressure. This is an example of the pressure-reversal of anaesthesia which is widely observed across a huge range of species and ought to provide some clue as to the mechanism of general anaesthesia. Work described in this issue of *Nature* (page 56) uses this approach and comes up with some intriguing results which may implicate glycine-mediated synaptic inhibition in the action of anaesthetics.

In pharmacological terms, general anaesthesia seems to be a rather crude affair not involving very specific molecular receptors: large concentrations of anaesthetic are used and almost any gas, even an inert gas such as helium, will act as an anaesthetic if applied at sufficient pressure. A vital practical ramification of this is the narcosis suffered by divers who can be made semiconscious as a result of the anaesthetic effects of nitrogen in air when they reach depths at which the anaesthetic pressure becomes significant.

The traditional interpretation of this lack of specificity and the influence of pressure has been that the effect of anaesthetic has a relatively simple physical origin. Since anaesthetic potency correlates with solubility in fat, it has been held that the results are achieved by solubility of the gas in lipid membranes; swelling produces anaesthesia reversible by compressing the

membranes. Alternatively, the hydrophobic part of a specific protein could be the site of solution.

In this week's *Nature*, Smith *et al.* provide a significant step forwards in elucidating the mechanism of anaesthesia in more precise molecular terms. The key observation is a complete difference in response to pressure between anaesthetized tadpoles and shrimps with anaesthetics chloroform, ether, ethanol, halothane and sodium pentobarbitone. Thus, whereas the relative potencies of these anaesthetics in the two species are very similar, the influence of pressure is totally opposite in the two cases: shrimps show no sign of pressure-reversal, indeed pressure can potentiate anaesthesia, while in tadpoles, the effects of anaesthetic are reversed by the application of pressure in otherwise identical circumstances.

The authors' preferred explanation of this dramatic difference derives from the fact that crustaceans, such as the freshwater shrimp, have a nervous system which is insensitive to strychnine. This implies they do not use glycine as a neurotransmitter. Thus, it is possible that the site of the solution of an anaesthetic is a hydrophobic part of the glycine receptor protein. Support for this notion comes from the fact that the pharmacology of pressure, as elucidated from pressure-induced convulsions in mammals, closely parallels that of strychnine which is believed to act on post-synaptic inhibition mediated by glycine. □

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Mass extinctions and missing matter

SIR — The approximate 30-Myr periodicity that appears to exist in the record of mass biological extinctions and terrestrial impact cratering has been interpreted by Rampino and myself¹ as evidence of a quasi-periodic bombardment of the Earth by comets that have been perturbed into Earth-crossing orbits by close gravitational encounters of the Solar System with massive interstellar clouds of gas and dust. In this model, the underlying clockwork is the Solar System's vertical oscillation through the midplane of the Galaxy, towards which most of the massive interstellar clouds are concentrated.

The downward force exerted by the Galaxy will also perturb the Solar System's comet halo, but will not produce any periodicity. To see why, first observe that the Solar System's vertical orbit² lies wholly within the linear domain of the galactic force law^{3,4}, so that the vertical galactic acceleration is simply $K(z) = -4\pi G\varrho_0 z$, where ϱ_0 represents the local mass density of material near the galactic plane. It follows that the difference between the galactic acceleration of the Sun at galactic height z and that of a comet at a distance r from the Sun in the direction of the galactic plane is $K(z) - K(z-r) = -4\pi G\varrho_0 r$. By equating this small galactic tidal acceleration to the local acceleration of the comet towards the Sun, $-GM_\odot/r^2$, a 'tidal radius' for the comet halo is readily obtained: $r_t = (M_\odot/4\pi\varrho_0)^{1/3} = 1.5 \times 10^5 \text{ AU}$. (For comparison, the Galaxy's central force field and differential rotation impose a tidal limit⁵ of $3 \times 10^5 \text{ AU}$.) Because of its independence of galactic height, r_t differs from the tidal radius that is derived for an inverse-square galactic force law, valid very far from the galactic plane⁶. Thus, the comet halo, unlike a far-ranging globular star cluster⁷, never experiences a periodic 'gravitational shock' as it passes up and down through the galactic disk (contrary to ref. 8), although it does suffer a constant amount of compression in the vertical direction. For this reason, individual objects like interstellar gas and dust clouds seem to be needed as perturbers.

Could other dark perturbing objects also be important? The disk of our Galaxy probably contains a large quantity of 'invisible matter'. Kinematical studies have produced a range of estimates for the total mass density ϱ_0 in the solar neighbourhood (believed to be typical of the part of the galactic plane that is accessible to the Solar System) equal to $0.14\text{--}0.24 M_\odot \text{ pc}^{-3}$ (refs 3, 4, 9–12). Direct counts of observed stars, gas, and dust have turned up only $0.10\text{--}0.11 M_\odot \text{ pc}^{-3}$ (refs 4, 10), and so the missing disk material could amount to as much as $\sim 0.14 M_\odot \text{ pc}^{-3}$.

The effectiveness of any seen or unseen matter in perturbing the Solar System's comet halo depends only on the local mass

density of this matter, ϱ_0 . From equations (11) and (12) of Hills¹³, the mean time interval between random encounters of the Solar System with objects of mass density ϱ is given by $\tau_s = \varrho^{-1} a^{-2}_{\min} \text{ Myr}$, if ϱ is in units of $M_\odot \text{ pc}^{-3}$ and $a_{\min}$ in units of $2 \times 10^4 \text{ AU}$, where $a_{\min}$ is the minimum semimajor axis of those comets for which the loss cone is filled by an encounter. (This result depends on the impulse approximation and point-mass approximation, which turn out to be adequate assumptions for all cases considered here.) Hills' equations (13) and (19) (ref. 13) (with $n = 2$) provide the total number of comet collisions with the Earth during an encounter, $N = N_0 a^{-2}_{\min}$, where $N_0 = 4$ or $N_0 = 0.2$ depending on whether Everhart's¹⁴ or Weissman's¹⁵ rate of present-day comet impacts is assumed. In either case, the shower of comets¹³ lasts $\tau_1 = 11 a^{3/2}_{\min} \text{ Myr}$. Because the outer radius of the main comet reservoir probably lies at $\sim 2 \times 10^4 \text{ AU}$ (refs 13, 16–19), $a_{\min}$ is not likely to exceed 1.

For the Solar System's vertical motion within the Galaxy to significantly modulate the background comet flux arising from random showers, the most important perturbing objects must not only have a high mass density but must also be concentrated fairly strongly towards the galactic plane. The total comet flux into the inner Solar System is due to solar encounters with known stars ($\sim 0.05 M_\odot \text{ pc}^{-3}$) (ref. 4), known interstellar gas and dust clouds ($\sim 0.05 M_\odot \text{ pc}^{-3}$) (ref. 20), interstellar comets ($< 6 \times 10^{-4} M_\odot \text{ pc}^{-3}$) (ref. 21), and invisible matter ($\leq 0.14 M_\odot \text{ pc}^{-3}$). If the invisible matter consists of small (or cold) interstellar molecular clouds, which would probably be concentrated strongly toward the galactic plane like the known molecular clouds and the youngest stars ($\sim 0.04 M_\odot \text{ pc}^{-3}$) (refs 4, 20), then the total percentage of local material lying in the flat subsystem could be as high as 75%. Even without invisible matter, the total percentage would still be fairly high, 40%. In all cases, the encounter time τ_s would be acceptably shorter than the Solar System's galactocentric half-period, $P_{1/2} = (\pi/4G\varrho_0)^{1/2} \approx 30 \text{ Myr}$, and so galactic modulation could arise.

If, however, the invisible material consists of a large population of old, dead stars, with a relatively small galactic concentration, the percentage of local matter belonging to the flat subsystem could be as low as 17%. This would be insufficient to modulate the background comet flux into the inner Solar System. It might be argued that most of these dead stars are young objects, not yet kinematically dispersed far from the galactic plane. However, they would also have to be old enough to have migrated into the area between the star-forming spiral arms (so as to adequately cover the region of the galactic plane that

the Solar System traverses in its revolution around the galactic centre). Their ages would consequently²² need to lie in the narrow range $10^8\text{--}10^9 \text{ yr}$. Yet it is very unlikely that much of the invisible matter consists of dead stars; the space densities of faint white dwarfs²³ and of black dwarfs^{24–26} are observed to fall off rapidly with declining luminosity (at least to $M_v = 21$), while most neutron stars and stellar black holes are expected to be derivatives of massive main-sequence stars, whose space densities, at least today, are observed to be extremely low⁴.

If future observations reveal a large secondary maximum in the luminosity function of dead stars at a lower light level (perhaps because star formation has been very sporadic), the implications for the present hypothesis of Galaxy-modulated comet impacts on Earth¹ could be profound.

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The case against impact extinctions

SIR — Some people think that the impact hypothesis for the mass extinction at the Cretaceous–Paleogene boundary is 'proved'^{1,2}. So it is, if one considers selectively one set of evidence. However, it is equally convincingly disproved if one considers selectively another set of evidence. I have recently reviewed this topic³, evaluating the literature through 1983; no valid later work known to me materially affects any of the many arguments in each direction

given in that paper and only one adds a useful new argument. The best evidence in each direction does not impinge on that for the other, and neither side has a satisfactory explanation for the opposing evidence.

The arguments against the effect of an impact, all discussed in my review, are:

- (1) A *Protunquatum* community lacking dinosaurs.
- (2) Appearance and progressive evolution of the *Protunquatum* community just before the boundary.
- (3) Freshwater community unaffected.
- (4) Last occurrence of dinosaurs being detectably below boundary in Montana and vicinity
- (5) Existence of transitional floras below boundary.
- (6) Putatively extraterrestrial material below boundary.
- (7) Absence of effects of elimination of stratospheric ozone.
- (8) Coincidence with culmination of very large regression.
- (9) Marsupials but not placentals nearly eliminated; most arboreal multituberculates and birds survived.
- (10) Darkness expected to occur too often.
- (11) Concentration of Ir, etc., in reduced material, with associated timing anomaly.
- (12) Dinosaurs occurring well above the palynological boundary in New Mexico.
- (13) Effect of predicted cooling not seen.
- (14) Effects of predicted acid rain not seen.
- (15) Absence of turbidites at boundary (if marine impact) and apparent absence of large terrestrial crater.

The first two items refer to a community of Paleocene aspect, whose members were ancestral to much of the Paleocene fauna and which appeared in Montana 4×10^5 yr before the boundary. Smit and van der Kaars⁵ claim that the sediments involved postdate the boundary, but their work ignores the field relationships given in a detailed geological map⁶ (other evidence and will be refuted more adequately in a paper in preparation by several people). That my list of evidence is one-sided, reflects merely the greater publicity which given to evidence favouring an impact as the cause of the extinction.

The results of Bohor *et al.*⁴, who found quartz fragments at the boundary which had been shocked at very high pressure, are at first sight strongly favourable to an impact. But until possible alternatives such as a uniquely powerful explosion (perhaps analogous to the smaller ones of kimberlites) are considered, some scepticism seems appropriate. Unless there were multiple impacts², for which there is no evidence (despite considerable search) except an unconvincing³ interpretation of osmium isotope ratios⁷, it is not apparent how the impact hypothesis can be modified to resolve the major conflicting evidence. Yet volcanism, even broadly conceived, has equally severe problems^{3,4}.

Coincidence is not a satisfactory basis for theory.

On the "late Eocene mass extinction", I know of no evidence that it exists. For instance, there are as many last appearances of marine families recorded in the middle Eocene as in the late Eocene⁸, neither value being unusually large; species extinctions are not concentrated at one horizon⁹; and the mammalian turnover is later⁹ and clearly involves at least a major biogeographic interchange¹⁰.

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Did komatiitic lavas erode channels on Mars?

SIR — Huppert *et al.*¹ show that terrestrial Precambrian komatiitic lavas may have erupted as very hot, highly fluid, turbulent flows capable of eroding deep channels and melting and assimilating rocks over which they flowed. We have argued² that the chemical composition of martian fines, measured by Viking Lander spacecraft³, indicate derivation from mafic igneous rock, that terrestrial Precambrian komatiitic lava would be a good candidate for an analogue. Using our derived Mars composition, Schonfeld⁴ calculated that martian lavas had viscosities of about 0.5 Pa s. The new findings of Huppert *et al.*, that komatiitic lavas could have been as hot as 1,700°C and as low in viscosity as 0.1 Pa s, renew our interest in the possibility of martian channel formation by lava⁵.

Various channels on Mars have been ascribed to tectonism, earthflows, wind action, glacial ice, and flowing basaltic lava, but the majority have features (braided channels, terraced walls, sculpted islands, dendritic tributaries, meanders, increase in size downstream) that have forced most observers to conclude they were formed by running water. However, the source of the water, and where the water is today, remain vexing problems. (Most workers believe that water had to be more abundant on Precambrian Mars and what water remains is locked principally in ground ice). Most of the previous objections to lava as the principal erosive agent on Mars (see ref.6) are based on observed characteristics of basalt. Such lavas erupt at around 1,200°C and have

viscosities more than fifty times that found by Huppert *et al.* for komatiites. If martian lavas were komatiitic, however, a water-like fluid would have been available and such a fluid would explain not only channel formation, but also the enormous lateral extent of martian flows and the absence of apparent outflow deposits (sediments) in the basins into which the large channels discharge. Finally, if water did not carve martian channels, the complex schemes to explain the present lack of surface water, including gross changes in the atmosphere/climate of the planet, are not needed.

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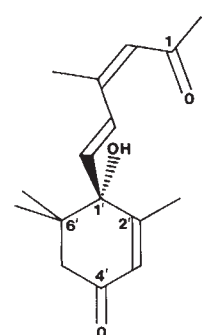
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A question of chirality

SIR — Hanke writes (*Nature* **310** 272; 1984) "It is quite remarkable that immunoglobulins should be able to discriminate between the enantiomers because, as can be seen from the diagram, the molecule of abscisic acid is almost symmetrical..."



To my eye, the degree of chirality shown by abscisic acid is almost exactly the same as that between my feet. Perhaps Hanke is more supple than me, but I cannot put my right foot into a left shoe. Why should a globulin be more flexible?

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

Origins of biomolecular handedness

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Classical mechanisms proposed for the transition from racemic geochemistry to homochiral biochemistry in terrestrial evolution generally ascribe to chance the particular handed choice of the L-amino acids and the D-sugars by self-replicating systems. The parity-violating weak neutral current interaction gives rise to an energy difference between a chiral molecule and its mirror-image isomer, resulting in a small stabilization of the L-amino acids and the L-peptides in the α -helix and the β -sheet conformation relative to the corresponding enantiomer. The energy difference suffices to break the chiral symmetry of autocatalytic racemic reaction sequences in an open non-equilibrium system.

THE class of handed or enantiomorphous molecules (see Fig. 1) was first identified in 1848 by Pasteur¹ from his study of optical activity, the rotation of the plane of polarized light by natural products in the fluid state or in solution. As a student, Pasteur found that the crystals of the sodium ammonium salt of an optically inactive tartaric acid, then termed racemic acid, are separable into two morphological classes, distinguished by hemihedral crystal facets which, in the one set, have a non-superposable mirror image relation to the corresponding facets of the other set of crystals. One set proved to be isomorphous with the sodium ammonium salt of the major naturally occurring isomer, (+)tartaric acid, and gave the same positive specific optical rotation in aqueous solution. The other set, morphologically enantiomorphous, gave a specific rotation of the same magnitude but negative in sign, and liberated the optical isomer, (–)tartaric acid, on acid treatment.

From the view then generally prevailing (see ref. 2), that a crystal and its constituent molecules are morphologically 'images of each other', Pasteur¹ concluded that the individual (+) and (–)tartaric acid molecules are structurally dissymmetric, related as non-superposable mirror-image forms, like the macroscopic hemihedral crystals of the corresponding sodium ammonium salts. While still current in France,³ Pasteur's term *dissymétrie* became generally supplanted elsewhere by Kelvin's expression 'chirality'⁴ from the familiar analogy of the morphological mirror-image relation between the left and the right hand.

Pasteur soon came to the view that chiral molecules, characterized by their fluid-state optical activity, and hemihedral crystals are the product of universal dissymmetric influences, exemplified by the polar fields of electricity and magnetism, or composite rotations, such as those of the inherently-chiral solar system⁵. Following the discovery by Faraday⁶ of magnetically-induced optical activity in isotropic transparent media, Pasteur grew normally holohedral crystals in a magnetic field with the object of inducing hemihedral crystal forms. Not discouraged by the negative results, Pasteur attempted to induce optical activity in synthetic products by running reactions in a centrifuge, and to modify the optical activity of natural products by rotating the plants producing them with a clockwork mechanism. These experiments, and their negative results, were reported by Pasteur^{5,7} in his later years (1884), in the course of a restatement of his views on the universality of dissymmetric forces and structures in nature. Previously, in his lectures on molecular dissymmetry, Pasteur⁸ (1860) had proposed that optical activity provides a demarcation criterion between the products of laboratory synthesis and the natural products synthesized biochemically by living organisms.

Chiral synthesis

Le Bel⁹ (1874), in his pioneer work parallel to that of van't Hoff¹⁰ on the connection between optical activity in the fluid phase and enantiomorphous three-dimensional molecular structures, proposed forces of dissymmetry additional to those considered by Pasteur. The reactions of symmetrical substrates, whether achiral or a racemic mixture of enantiomers, carried out photochemically with left- or right-circularly polarized electromagnetic radiation, or thermally with a chiral catalyst, would be expected to produce an excess of a particular enantiomeric product⁹.

The differential absorption of left- and right-circularly polarized light by chiral molecules in solution, isotropic circular dichroism, was discovered by Cotton¹¹ (1895). It provided a physical basis for the proposed chiral photodiscrimination, which however eluded Cotton¹² himself. The first unambiguous photoresolutions of fluid racemic mixtures were achieved by Kuhn and co-workers^{13,14} (1929), and the first chiral photosyntheses from achiral precursors, notably, the helicene enantiomers^{15–17}, are relatively recent.

The second of the dissymmetric influences on reactivity considered by Le Bel⁹, the use of chiral reagents in thermal reactions, was implicit in Pasteur's¹⁸ (1853) discovery of diastereomers. The introduction of a second chiral centre into an enantiomer gives two diastereomers with different chemical and physical properties, apart from optical activity. In general, the yields of the two diastereomers are unequal, as Fischer¹⁹ (1894) showed in the step-wise synthesis of the D-series of sugars from (+)glyceraldehyde, and the L-series from the corresponding (–)enantiomer. Fischer¹⁹ found the reactions of diastereomers mediated by naturally-occurring chiral catalysts to be particularly selective, showing that α -methyl-D-glucoside is hydrolysed by maltase, but not by emulsin, whereas β -methyl-D-glucoside is cleaved by emulsin, but not by maltase, although neither of the corresponding L-glucosides are affected by either enzyme preparation.

The stereochemical 'key and lock' hypothesis of Fischer^{19,20}, based upon these and analogous observations, went far to account for the substantial homochiral biochemistry of living organisms, the general adoption of only one of the two enantiomeric series of the α -amino acids and, equally, of the sugars. Subsequent developments of the 'key and lock' hypothesis suggest a more dynamic relation, a mutual stereochemical adaptation between the catalyst and the substrate. Pauling²¹ proposed that the catalytic activity of an enzyme derives from the steric distortion of the substrate when it binds, towards the structure

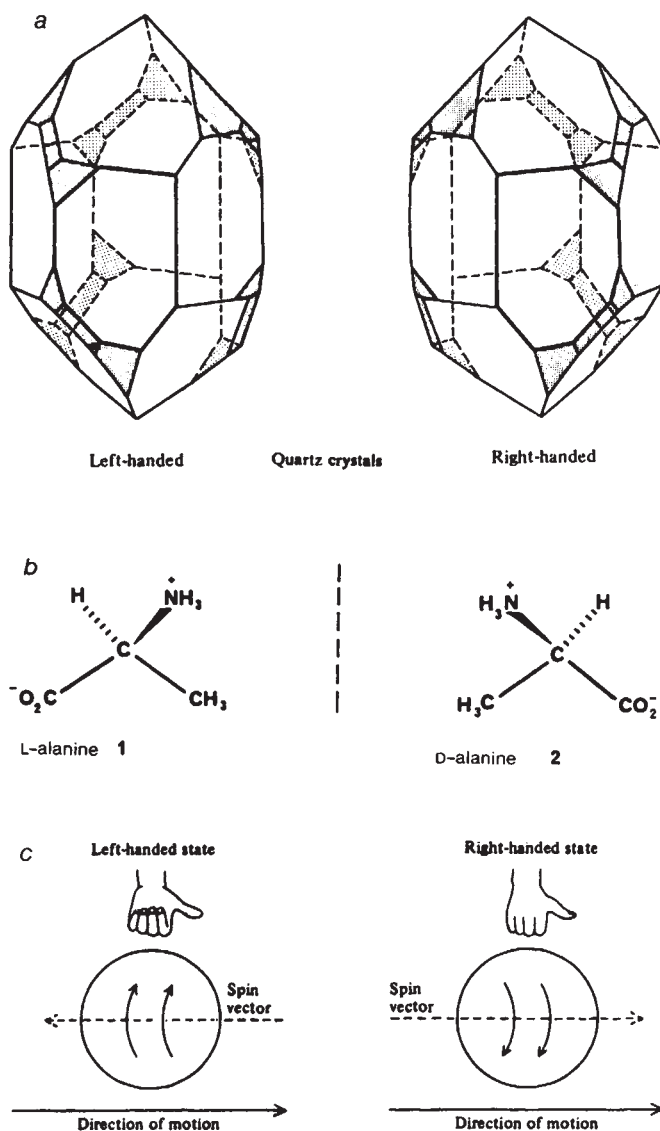


Fig. 1 A chiral or handed structure is not superposable upon the corresponding mirror-image form, its enantiomer. Chirality appears macroscopically (a), as in quartz crystals, in which the minor crystal facets follow either a left-handed or a right-handed sequence when viewed in a direction along the three-fold crystal axis. At the molecular level, chirality is exhibited by organic compounds (b), such as L-alanine (1) and D-alanine (2), in the respective left-handed (anticlockwise) or right-handed (clockwise) sequence of the NH_3^+ , CO_2^- , and CH_3 groups attached to the central carbon atom, when viewed with the bonded hydrogen atom remote from the observer. In the case of a particle (c), such as an electron, the left-handed or right-handed chirality derives from the respective antiparallel, or parallel, relation between the linear momentum vector and the angular momentum axial vector.

of the transition state for reaction, a view supported by X-ray structural studies of crystalline enzyme-substrate complexes and by a range of related fluid-state investigations²².

The results of kinetic studies and product analyses of the synthesis and hydrolysis of polypeptides provide further grounds for the adoption of a homochiral biochemistry by living systems. The polymerisation of a given enantiomer of an α -amino acid, in the form of the *N*-carboxyanhydride derivative, proceeds at a rate faster by a factor of ~ 20 than that of the corresponding racemic mixture in the same conditions²³. The partial polymerization of near-racemic mixtures of D- and L-leucine-*N*-carboxyanhydride, containing a small excess of one of the enantiomers, gives a polyleucine in which the original enantiomeric excess is enhanced and, when the polymer product

is partially hydrolysed, the degree of homochirality in the residual polymer is further increased²⁴.

Although the general stereochemical and energetic bases, if not the detailed mechanisms, are clear for the natural selection of an efficient homochiral biochemistry by living organisms, the particular choice of the L-amino acids and the D-sugars appears generally to be a matter of chance²⁵. The classical dissymmetric influences, based on electromagnetism or gravitation and the Coriolis force of rotation, might have equally generated an initial enantiomeric excess of D-amino acids or L-sugars which propagated to an enantiomeric homochiral biochemistry.

Chiral fields

In an application of his general principle, that the symmetry of a physical effect implies a corresponding symmetry in the causes, Curie²⁶ pointed out that each of the polar fields which Pasteur had taken to be dissymmetric has mirror symmetry individually. Any type of chiral field is necessarily composite, with collinear axial and polar vector components, or corresponding higher-rank tensor components. The simultaneous application of a magnetic field and a parallel or an antiparallel electric field provides such a chiral combination, the parallel relation being transformed into the enantiomeric antiparallel relation by mirror-reflection or, equivalently, by space inversion.

Curie²⁶ conjectured that the synthesis of an optically-active substance from an achiral substrate might be feasible by running the reaction in a chiral combination of an electric and a magnetic field. The conjecture was refuted, at least for the case of uniform static fields, by applications²⁷⁻²⁹ of Wigner's general principle³⁰, that a complete experiment subject to space-inversion and time-reversal should give a transformed experiment which is possible in principle. The application of the parity operator, space-inversion, to a chiral molecule in parallel, static, and uniform electric and magnetic fields transforms the molecule into its mirror-image isomer and the fields into the enantiomeric antiparallel combination. The reversal of the direction of the circular electric current producing the magnetic field, equivalent to time-reversal, leaves the mirror-image isomer unchanged but restores the parallel relation of the electric and the magnetic field. The cycle implies that the fields do not discriminate between a chiral molecule and its enantiomer, both substances having an identical energy in the same combination of environmental fields, so that a racemic mixture inevitably results from a reaction carried to equilibrium in the field combination.

Similar conclusions are derived for the chiral products of reactions run in a centrifuge rotating clockwise or anticlockwise horizontally in a vertical gravitational field^{28,29}. The simultaneous application of three uniform fields, electric, magnetic, and gravitation, for example, is required to discriminate between the enantiomeric products from achiral reactants. The discrimination with realistically attainable fields is, however, vanishingly small^{28,29}. The most significant of the classical chiral fields is that of circularly polarized electromagnetic radiation, but the discrimination is even-handed, and the pure electromagnetic circular dichroism of a D-isomer, while opposite in sign, is of equal magnitude to that of its L-enantiomer.

Parity non-conservation

A group of anomalies in elementary particle physics led Lee and Yang³¹ to conclude that parity, space-inversion equivalence, is not conserved in the weak nuclear interaction, contrary to the original principle of Wigner³⁰. A consequence of parity violation, the asymmetric β -decay of radionuclides, was observed in the decay of cobalt-60 to nickel-60 with the emission of an electron³², and in the emission of the corresponding antiparticle, the positron³³, in the decay of cobalt-58 to iron-58. These and other studies of the weak interaction establish an intrinsic left-chirality for the electron, antiparallel momentum and spin vectors, and right-chirality for the positron, parallel momentum and spin vectors, in proportion to the ratio of the velocity of the particle, or the antiparticle, to the velocity of light.

The discovery of parity non-conservation in the weak interaction led to the chemical expectation that the β -decay of nucleides should give rise to an enantio-differentiating radiolysis of racemic mixtures, or to the corresponding photolysis by the circularly-polarized electromagnetic radiation arising from the progressive retardation of the emitted spin-polarized electron or positron³⁴. As yet, no unequivocal enantio-selective radiolyses of racemic mixtures have been reported by the agency of β -radionucleides³⁵, or of spin-polarized electrons³⁶ or spin-polarized positrons³⁷ produced by particle accelerators.

The weak interactions initially investigated, notably β -decay, are mediated by the charged massive bosons, $W^\pm$, in the weak charged currents, which have only a minor, high-order, significance for charge-retaining atomic and molecular properties. An additional weak neutral current interaction, involving the massive neutral boson, Z^0 , recently detected, together with its charged counterparts, $W^\pm$, at CERN³⁸, emerged from the unification of the weak with the electromagnetic interaction by Weinberg³⁹ and Salam⁴⁰. The weak neutral current (WNC) is more significant for charge-retaining properties of atoms and molecules, including the stationary state properties, as the perturbation of the state properties by the parity-violating electro-weak interaction between each valence or inner-shell electron and the nucleus or other electrons of an atom remains finite in the non-relativistic limit.

Due to the WNC interaction, all atoms and achiral molecules, as well as enantiomers, are expected to be optically active to electromagnetic radiation and to polarized matter waves. Further, enantiomeric molecules have different electronic binding energies, owing to the dependence of the WNC interaction between different atomic centres on the particular handedness of the molecular stereochemistry. Just as the electron and the positron have intrinsic enantiomeric chirality, so a D-isomer composed of electrons and other particles has a true isoenergetic mirror-image L-enantiomer made up of positrons and other antiparticles in a hypothetical counter-world of antimatter, but the natural particulate L-isomer is not energetically equivalent⁴¹.

The optical rotation of electromagnetic radiation by a free atom, due to the WNC interaction, is expected to be proportional to Z^6 , where Z is the atomic number^{42,43}. Accordingly, vapours of heavy metal atoms have been investigated, and an optical rotatory dispersion of the correct sign and order of magnitude has been reported for electronic transitions of atomic bismuth, lead, thallium and caesium in the vapour phase⁴²⁻⁴⁴. Similarly, the matter wave analogue of optical rotation, due to the WNC interaction, has been observed in the rotation of the spin-polarization of a cold neutron beam by crystals of metallic tin, the tin-117 isotope giving a particularly large rotation⁴⁵.

The WNC interaction in chiral molecules gives rise to a parity-violating energy shift, E_{pv} , which is positive for one isomer and negative for its enantiomer. Early estimates^{46,47} gave a magnitude order, $E_{pv} \sim 10^{-12}$ eV, based upon a crystal field model where a single heavy atom is perturbed by a chiral electrostatic field. More fundamental estimates of E_{pv} , based upon the molecular orbital wavefunctions, are substantially smaller for chiral molecules composed of light atoms⁴⁸. The wave functions of a model chiral ethylene molecule, twisted through 10° to dihedral symmetry⁴⁹, provide an energy shift, E_{pv} , of $\sim \pm 5 \times 10^{-19}$ eV, positive for the (R)-isomer and negative for the (S)-enantiomer⁴⁸.

Ab initio calculations on a simple chiral molecule, hydrogen peroxide⁵⁰, show that the energy shift, E_{pv} , is sensitive to the molecular conformation, with an optimum value of the same magnitude, $\sim \pm 5 \times 10^{-19}$ eV. The energy shift, E_{pv} , for the α -amino acids is also sensitive to the molecular conformation and, at the conformation preferred in aqueous solution, L-alanine is more stable than the D-enantiomer by $\sim -6 \times 10^{-19}$ eV. The extension of the calculations to a peptide unit of a polypeptide in the α -helix or the β -sheet conformation shows that the polypeptides from the L-amino acids are stabilized relative to the corresponding D-enantiomers in both conformations by $\sim -9 \times 10^{-20}$ eV for each peptide unit^{51,52}.

These parity-violating energy differences between enantiomers amount to no more than $\sim -2 \times 10^{-14}$ J mol⁻¹ per amino acid residue in the polymer, corresponding to an enantiomeric excess of some 10^6 L-peptide molecules per mole of racemate in thermodynamic equilibrium at ambient temperature. Although small, the enantiomeric excess indicates a determinate intrinsic bias in organic nature for the particular handedness which evolved historically into homochiral dominance. The sign and the magnitude of the parity-violating energy shift for a sugar enantiomer remains undetermined as yet. Chemically, however, the D-sugars are connected with the L-amino acids through the conversion of D-glucosamine to L-alanine⁵³.

Chiral symmetry breaking

Observable consequences of the parity-violating energy difference between enantiomeric molecules, owing to the small magnitudes involved, require either an extended, cumulative, amplification mechanism, or an open, dynamic, metastable system with a selection between alternative outputs triggered by a very small perturbation. Systems of the latter type involve the catastrophe theory of symmetry-breaking, in which ordered dissymmetry emerges from an open non-equilibrium process⁵⁴. Kinetic reaction sequences designed to produce a spontaneous transition from a racemic reaction process to the one or the other of the two constituent homochiral reaction sequences postulate autocatalytic branched-chain kinetics in each reaction channel, accompanied by competitive cross-reactions between the two enantiomeric sequences that are mutually inhibitory and form inactive products⁵⁵⁻⁶¹. An open reaction scheme proposed recently has the general form⁵⁹,



and,



In an open non-equilibrium system with an input of achiral substrates A and B, and an output composed of the enantiomers L and D, together with the inactive product P, formed irreversibly, the overall process remains racemic if corresponding primed and unprimed rate constants are equal and the input remains small. As the input concentrations of A and B are increased, the racemic process becomes metastable and switches spontaneously into the one or the other enantiomeric homochiral reaction sequence, (1a) and (2a) or (1b) and (2b), breaking the racemic chiral symmetry⁵⁹. In a collection of such systems, it is expected that the L-channel would be adopted by one half of the reaction sequences and the D-channel by the other half.

At the onset of racemic metastability in the reaction sequence (1), (2) and (3), the bifurcation point dependent on the substrate input, the system becomes hypersensitive to small chiral perturbations, which determine the particular homochiral channel adopted by the system. The chiral perturbation may be external to the system, or intrinsic, such as a small difference, ΔE , between the activation energy of corresponding primed and unprimed reactions in the processes (1) and (2). Thermal fluctuations, which increase with a rise in temperature, offset the hypersensitivity of the reaction sequence at the bifurcation to chiral perturbations, and the overall chiral discrimination governing the choice of a homochiral channel becomes proportional to $(\Delta E/kT)$. The critical magnitude order, $(\Delta E/kT) \geq 10^{-17}$, is

found⁵⁹ to govern the selection of a particular enantiomeric reaction sequence in the series (1), (2) and (3).

At ambient temperature, the estimated parity-violating energy differences between enantiomers composed of light atoms correspond to the lower limit of the criterion, $(E_{pv}/kT) \sim 10^{-17}$. The determinate selection of the lower-energy homochiral reaction sequence is promoted by a slow passage through the transitional point where the racemic reaction process bifurcates into the two homochiral reaction channels, that is, by small increments of the input concentrations of the substrates A and B, extended over long time periods. From the parity-violating energy differences between the enantiomers of the α -amino acids and of the polypeptides, it is estimated that the selection of the lower-energy L-series occupied a minimum time of $\sim 10^3$ years⁶¹.

An external chiral perturbation, ΔE_{ext} , of the reaction sequence (1), (2) and (3), made up from the combination of three macroscopic vector fields, electric, magnetic, and gravitation, does not meet the above mentioned criterion because, even with extreme field strengths, the ambient temperature ratio amounts to no more⁵⁹ than $(\Delta E_{ext}/kT) \sim 10^{-19}$. The external perturbation of circularly polarized light is not eliminated however. While the pure electromagnetic circular dichroism of two enantiomers is equal in magnitude and opposite in sign, the electro-weak interaction gives each atom in the two molecules a small additional optical activity which is of the same sign for each enantiomer. The magnitude-symmetry of the differential absorption of left and right circularly polarized light by enantiomeric molecules is broken by the WNC interaction, and even equally alternating opposite circular components in the solar radiation would tend to favour one of the two enantiomeric reaction channels in the long run. Owing to multiple aerosol scattering, twilight is $\sim 0.1\%$ circularly polarized, particularly in the near infrared region, with one circular component in excess at sunrise and the other at sunset⁶².

A uniformitarian model, alternative to that of the catastrophic bifurcation of competitive reaction sequences, depends upon the progressive accumulation of small differences between each successive enantiomeric stage in a manifold chemical or physical process, starting from an achiral or racemic substrate and terminating with an enantiomerically enriched or homochiral product⁶³⁻⁶⁹. The accumulation model, proposed initially to account for the prevalence of homochiral biopolymers among the natural products⁶³, postulates that a small difference between the activation parameters of the enantiomeric monomers, ΔE , effective in relation to the thermal fluctuations, kT , through the ratio, $\epsilon = (\Delta E/kT)$, at each of the n stages of the polymerization, results in an excess of one of the homochiral n -fold polymers. The relative enantiomeric excess of the favoured n -fold polymer, $L(n)$, say, is dependent upon the degree of polymerization, n , and the advantage ratio, ϵ ,

$$[L(n) - D(n)]/[L(n) + D(n)] \approx n\epsilon \quad (4)$$

Subsequent applications of the accumulation model include cases of the spontaneous optical resolution of racemic substances by crystallization⁶⁶⁻⁶⁹. By the use of the relation (4), the advantage ratio is estimated at $\epsilon \sim 10^{-10}$ from the distribution of (+) and (-) crystals formed in 63 recrystallizations of racemic sodium ammonium tartrate⁶⁹, compared to a value, $\epsilon \sim 10^{-6}$, obtained from the polymerization of monomeric alanine derivatives⁶⁴. Both values are substantially larger than the minimum ratio, $\epsilon \sim 10^{-17}$, required by the bifurcation model⁵⁹, and approximately equal to the ambient temperature ratio, E_{pv}/kT , for the parity-violating energy difference between enantiomers composed of the lighter elements⁵⁰⁻⁵².

The condensation of optically-impure monomeric α -amino acid derivatives gives heterochiral polymers^{23,24}, not the homochiral polymers as required by the progressive accumulation model⁶³. Further, no equilibrated racemate is expected to be truly equimolecular in its constituent enantiomers, owing to the parity-violating energy difference between them, so that neither the polymerization of enantiomeric monomers, nor an

optical resolution by crystallization, begin with exactly equal enantiomer concentrations. Synthetic racemic camphor is reported to contain an excess of up to 3 or 4% of (+)camphor⁶⁸, and, after four successive recrystallizations of racemic tartaric acid, a minor excess of the (+)isomer was found to remain in the final crop of crystals and in the last of the mother liquors⁷⁰.

In a general criticism of the progressive accumulation model, it is argued that the condensation of an exactly equimolecular mixture of enantiomeric monomers would necessarily terminate with an equimolecular mixture of homochiral n -fold polymers, the enantiomeric enrichment of the incomplete process being transient⁵⁸. The objection is applicable to a closed system of optically-stable enantiomers in a racemic process, but not to an open system, with a continuous input of substrate and output of chiral product, nor to a closed system in which a stable chiral product forms from an achiral or optically-labile substrate.

Sometimes, the formation of enantiomorphous crystals by an achiral substance, for example sodium chlorate⁷¹, or an optically-labile racemate, for example allylethylmethyl-anilinium iodide⁷², gives, in a single experiment, crystals predominantly of one hand. As a result of many experiments, involving 46 crystallizations and 3,206 crystals in the case of sodium chlorate⁷¹, the weighted mean of the number of (+) and (-) crystals approaches equality. However because the departure from equality attains the 90% confidence level, it is not a matter of chance in the case of the terrestrial distribution of quartz where, over a total collection of 16,807 crystals, a 1% enantiomeric excess of (-)quartz is documented⁷³. The (-)quartz bias is common to all the locations sampled in the Americas, Europe, and Asia, and the collection excludes 931 unrepresentative sets of crystals, in which the mean enantiomeric excess of (-)quartz rises to 9.8%.

The excess of (-)crystals in the quartz laid down in the post-biotic period may arise from chiral biomolecular influences, although the elevated temperatures favouring the hydrothermal crystallization of quartz do not support this. While L-aniline is preferentially absorbed from the corresponding racemic mixture by (-)quartz^{74,75}, this and other α -amino acids are racemized at 142 °C in aqueous solution with a half-life of no more than a few days⁷⁶. If a significant proportion of crystalline quartz was laid down in the prebiotic era, the excess of (-)quartz provides not only a possible perturbation of determinate chirality in the bifurcation or the accumulation model for the generation of biomolecular homochirality, but also evidence for a small intrinsic parity non-conservation in the physical world at large.

Conclusion

The significance of the electro-weak interaction is established in particle physics, through the detection of the massive neutral boson, Z^0 , mediating the weak neutral current³⁸, and in atomic physics, by measurements of the optical activity of heavy metal atoms⁴²⁻⁴⁴. The importance of the WNC interaction for chiral systems, particularly biomolecules, is confined as yet to small enantiomers, composed of the lighter elements, for which reliable *ab initio* computations are currently feasible. The calculations to date of the parity violating energy difference between the enantiomers of the α -amino acids and the polypeptides in the α -helix and the β -sheet conformation provide a basis for the viewpoint that the adoption of the L- α -amino acid series, at least, in the homochiral biochemistry of living organisms was fully determinate, and not merely a matter of chance.

The current calculations refer to the range of molecular conformations stereochemically permitted for a small enantiomer in its electronic ground state. Corresponding computations of the potential energy surfaces for the stereoisomeric transition states connecting an archiral set of substrates with an enantiomeric pair of products are required, for example the reaction surfaces linking an aldehyde and ammonia or hydrogen cyanide with the enantiomeric aldehyde-ammonia or cyanohydrin products. These component reactions make up the traditional

Strecker synthesis of α -amino acids from carbonyl compounds, a synthesis which is considered to be significant in biogeochemical evolution. The evaluation of the potential energy surfaces for enantiomeric reactions, including the parity-violating energy shift, has importance for both the bifurcation and the accumulation mechanism proposed for the emergence of biochemical homochirality.

As yet, an open, flow-reactor, reaction sequence, involving the autocatalytic chain-branching of enantiomeric intermediates, has not been devised for an experimental investigation of the triggered bifurcation model for the spontaneous development of homochiral from racemic reaction systems. The nearest analogous reaction sequences characterized, although rather remote, involve the autocatalytic chain-branching of atomic radicals and other achiral intermediates, notably, the oscillatory chemical reactions of the Belousov-Zhabotinsky type, in which the reaction system switches spontaneously from one quasi steady state to another^{54,77-79}.

The alternative accumulation model for the emergence of homochirality from racemic systems is the more readily testable in principle at present. The practical limitation of the investigation of racemic monomer condensations, namely, the formation of heterochiral polymers, has an analogue, the formation of heterochirally twinned crystals, in studies of the spontaneous optical resolution of racemates by crystallization. The heterochiral Brazil twinning of quartz crystals provides an example, although the twinning is readily identified and the homochiral crystals are the more generally prevalent⁷³.

A study of the relative abundance of the (+) and the (−) crystals formed by solutions of an optically-labile racemate, or an achiral substance, affords an immediately practicable method of investigating the parity-violating energy difference between enantiomers, due to the electroweak interaction. Although the energy difference is small, it is multiplied by the number of molecules, or the number of unit cells, in a macroscopic crystal, as prescribed by relation (4). Further, the parity violating energy difference between enantiomers is expected to be proportional to Z^5 , where Z is the atomic number of the heavy atom or atoms in the molecule^{47,48}, and there are indications, from the *ab initio* calculations of the energy differences carried out to date, that such a proportionality holds to a fair approximation⁵².

Reports in the literature describe the formation of predominantly dextro-rotatory crystals from aqueous solutions of potassium dodecatungstosilicate⁸⁰⁻⁸², and of the corresponding dodecamolybdosilicate with a smaller, but still substantial, enantiomeric excess⁸². A similar substantial excess of dextro-rotatory crystals is reported for 21 recrystallizations of allylethylmethyl-anilinium iodide⁷². These salts contain relatively heavy elements, Mo ($Z = 42$), I ($Z = 53$), and W ($Z = 74$). The heaviest element readily available, uranium ($Z = 92$), provides, in the form of sodium uranyl acetate, which crystallizes⁸³ in the chiral cubic space group, P2₁3, a promising material for an investigation of the parity-violating energy difference between enantiomorphous structures due to the electro-weak interaction from the relative and absolute abundance of (+) and (−) crystals formed in a spontaneous optical resolution by crystallization.

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A general circulation model of water isotope cycles in the atmosphere

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H₂¹⁸O and HDO are important climatic tracers largely used to reconstruct continental palaeoclimates. The only thorough way to compare isotopic distributions in precipitation for modern and palaeoconditions is to simulate isotope cycles using atmospheric general circulation models. H₂¹⁸O and HDO cycles incorporated into our atmospheric general circulation model show good agreement between observed and predicted values for modern conditions.

THE water isotopes H₂¹⁸O and HDO are important climatic tracers for several reasons. First, on a worldwide scale, ¹⁸O and deuterium contents of precipitation decrease with the mean surface-air temperature at the sampling site¹; this relationship can be used to reconstruct palaeotemperatures, especially at high latitudes where it is particularly well obeyed²⁻⁵. Second, the relationship between ¹⁸O and deuterium contents of precipitation, $\delta D - 8\delta^{18}O = d$ (where d is 10‰ at present⁶, with $\delta^{18}O$ and δD expressed in ‰ versus the standard mean ocean water, SMOW, reference), appears to be climate dependent, with smaller values of deuterium excess d during the last ice age (18,000 yr BP)^{7,8}; the smaller ice age values found in Antarctic ice cores have been interpreted⁷, using a simple model, as reflecting higher relative humidity over the corresponding moisture source regions. Third, spatial and temporal variability of isotope contents of precipitation depends not only on temperature, but also on other atmospheric or geographical parameters¹, such as intensity of precipitation and air mass origin; these further dependencies make climate reconstructions more difficult, but at the same time could be useful as additional climatic information.

Simple models have been derived to study the dependency of isotope contents of vapour and precipitation on atmospheric parameters. These models take into account all the details of fractionation processes but they only deal with prescribed trajectories of isolated air masses. They yield results consistent with the mean temperature-isotope and $\delta D - \delta^{18}O$ relationships^{1,9,10} and can also be used to study more complex dependencies, such as amount of precipitation, air mass origin or meteorological path¹¹. The only way, however, to reconstruct the full space-time variations of isotope contents of vapour and precipitation is to include the isotope cycle modelling in atmospheric general circulation models (AGCMs) which explicitly simulate the global and regional features of atmospheric dynamics and thermodynamics, with fully detailed hydrological cycles. Such AGCM modelling of isotope cycles must be tested first on modern conditions for which reasonably good documentation of isotope contents is available: vertical profiles have been measured¹²⁻¹⁴, spatial and seasonal distributions in precipitation are reasonably well known, mainly from the IAEA network¹⁵. Once it has been validated against modern data, AGCM isotope modelling can be used in palaeoclimatic simulations; this may be a first step towards a better interpretation of isotope changes from ancient to present times in terms of changes in atmospheric parameters.

Model description

The AGCM¹⁶ of the Laboratoire de Météorologie Dynamique (LMD) is an 11-layer gridpoint general circulation model with a regular longitude-sine-of-latitude grid, based on primitive equations (horizontal equation of motion, hydrostatic equation,

equation of continuity and thermodynamic equation). It includes a fully interactive hydrological cycle as well as prognostic equations for soil moisture, land- and ice-surface temperature and snow depth. Radiative fluxes are calculated taking into account model predicted cloud cover¹⁷. Orography, land and sea-ice extent, sea-surface temperature and ocean-surface albedo are prescribed external parameters; in the present experiments, land-surface albedo is a prescribed function of soil moisture and snow depth, based on the CLIMAP data set¹⁸.

The basic hydrological cycle of the LMD AGCM¹⁶ includes: surface evaporation over oceans and continents, vertical turbulent diffusion within the planetary boundary layer, horizontal and vertical transport, condensation processes, vertical diffusion in convective clouds and re-evaporation under cloud base. Within this basic hydrological cycle, differentiation between the water isotope species occurs at each phase change through fractionation processes resulting from differences in water vapour pressures and molecular diffusivities. Because HDO and H₂¹⁸O have lower vapour pressures than H₂¹⁶O, the condensed phase in equilibrium with vapour is enriched in heavy isotopes up to a fractionation coefficient α ($\alpha > 1$) (refs 19-21). On the other hand, differences in molecular diffusivities²² lead to kinetic fractionation which changes the relative behaviour of H₂¹⁸O and HDO for non-equilibrium processes.

Surface evaporation in the basic water cycle is proportional to a drag coefficient C_D , surface wind speed and the difference between the saturated mixing ratio at ground-surface temperature and the air mixing ratio at 10 m above the surface. Over land without snow or ice cover, surface moisture fluxes are limited through an evapo-transpiration coefficient which is a simple increasing function of soil moisture¹⁶. For H₂¹⁸O and HDO, the parameterization used for surface evaporation²³ differs over ocean, snow or ice, and land. At the air-ocean interface, water isotope fluxes are treated in the same way as the water vapour flux: that is, they are proportional to the difference between isotopic mixing ratio at ocean surface (in equilibrium with the ocean) and at 10 m. Isotopic drag coefficients are formulated according to Merlivat and Jouzel⁹ in order to represent kinetic effects. Over snow and ice, no fractionation is assumed because the time scale of isotope exchanges inside snow or ice crystals is large; therefore, water evaporates with the isotopic ratio of the ground surface. Over land without snow or ice, evaporation is also assumed to occur without fractionation. This assumption, which seemed reasonable from the experimental results of Zimmermann *et al.*²⁴, has also been used by Rozanski *et al.*²⁵; it was recently supported by a simple model and confirmed in studies under controlled environmental and natural conditions^{26,27}. Condensation, however, is not treated like evaporation (except over oceans): isotopic equilibrium is always assumed between condensed water and surface vapour.

The basic hydrological cycle uses two ground water reservoirs¹⁶, snow depth and soil moisture, whose evolution is governed by precipitation, surface evaporation or condensation, snowmelt and runoff. Soil moisture is defined as a single-layer parameter without differentiating surface and deep soil contents; runoff occurs whenever soil moisture exceeds a maximum value of 15 cm. Although the isotopic ratio of the soil moisture reservoir is not in reality constant with depth, we use a single ratio for the entire soil water reservoir²³, the evolution of which is governed only by precipitation and condensation, as surface evaporation, snowmelt and runoff have no fractionation effects. This 'mixed single layer' approach is, in fact, the simplest way to retain a memory of the past precipitation–condensation processes without using an expensive multilayer formulation. This simplification is, to some extent, equivalent to a kind of time averaging of precipitations; therefore, it is likely to yield a systematic underestimation of surface variability, and distortions of time lag effects (which may not be too important as long as seasonal variations are not considered). In the case of snow or ice cover, a similar 'mixed single layer' approach is used²³, although the molecular exchanges inside snow or ice are very slow. Furthermore, as there is no fractionation during snowmelt and surface evaporation, we do not distinguish snow and soil liquid water isotope contents. Over land ice, the single mixed-layer reservoir for snow and ice used for isotope budgets has an equivalent water depth of only 1 cm, in order to average precipitations over seasonal time scales. In the case of sea ice, the single reservoir is limited to the snow layer; over bare sea ice, we assume constant isotopic ratios²⁸: +3‰ for H_2^{18}O and +20‰ for HDO.

Like H_2^{16}O , the two isotope species HDO and H_2^{18}O are diffused vertically across the planetary boundary layer with a turbulent diffusion coefficient depending on static stability and vertical wind shear¹⁶.

Transport is a critical process in isotope modelling²³. Ordinary second-order finite difference schemes applied to the transport equation are not effective for treating positive quantities which may have large variations from one gridpoint to the next; in particular, they can produce unrealistic negative values. The problem is not very serious when only water vapour is advected, as negative values can be corrected *a posteriori*. When isotope species are simultaneously modelled, however, the occurrence of negative values must be avoided because of resulting dramatic inconsistencies in isotopic ratios (preliminary tests have shown that values up to 10⁸% are easily generated). In the present simulation, horizontal transport is modelled, using a first-order upstream flux form. All isotope species are, therefore, transported consistently in horizontal directions. The upstream scheme is strongly diffusive along the flow streamlines, but it has the advantage of producing more coherent precipitation patterns when it interacts with convective parameterizations. However, this upstream scheme cannot be applied in the vertical direction because it would lead to an unrealistically humid stratosphere. Vertical transport of water vapour is, therefore, modelled using a second-order flux form based on harmonic means between neighbouring points²³; this scheme is not diffusive and ensures positive values of the water vapour mixing ratio. The fluxes of HDO and H_2^{18}O are computed from the product of the water vapour flux and the arithmetic means of isotopic ratios. This technique ensures a consistent treatment of all isotope species and keeps isotopic ratios within reasonable bounds^{12,13}. More elaborate methods, such as 'flux corrected techniques'²⁹, should also be considered for future simulations.

The LMD AGCM distinguishes two kinds of condensation processes, large-scale condensation and small-scale convection¹⁶. The first case occurs when the air is supersaturated and the temperature lapse rate is less than the moist adiabatic lapse rate (non-convective clouds). Water vapour is condensed and temperature rises due to the release of latent heat until the mixing ratios reach the saturation value at the new temperature. Supersaturation is then tested downwards, starting from the top layer, and condensed water vapour is eventually re-evaporated

in the next layer down when the air is undersaturated. The second case occurs when the air is conditionally unstable, with a temperature lapse rate exceeding the moist adiabatic lapse rate (convective clouds). For supersaturated air, the moist convective adjustment technique³⁰ is used: the temperature is adjusted to the previous estimate of the moist adiabatic lapse rate, with energy being conserved in the process over the whole unstable range. The mixing ratios are then set equal to saturation values at the adjusted temperature and the excess precipitates. For unsaturated layers, condensation may occur in moisture convergence areas. Here we follow Kuo's parametrization³¹ of cumulus convection: we assume a moist adiabatic profile within the cloud with a moist static energy equal to its saturation value at cloud base, that is at the top of the mixed layer; the fractional area occupied by the cloud is determined as the ratio of latent heat available from moisture convergence at cloud base, to the energy necessary to generate the whole cloud on the mesh size. Temperature and humidity are then homogenized within the mesh, and the excess of moisture precipitates. In the present simulations, precipitating water re-evaporates under convective cloud base until the air becomes saturated.

At each phase change, isotopic fractionation is modelled²³ according to the type of phase change and the nature of the phase formed. Two phase transitions can lead to ice formation: direct deposition of vapour onto a solid phase, which actually occurs below -15°C , and the freezing of liquid droplets. In the first case, as the molecular exchanges are very slow inside solid crystals, the classical Rayleigh or open system model is assumed³²: the condensed phase is immediately removed after its formation and leaves the air parcel in isotopic equilibrium with the vapour phase. The second case occurs between -15 and 0°C without any isotopic fractionation, since, whatever their size, the freezing time of droplets and drops is negligible in comparison with their isotopic relaxation time³³. The case of liquid formation, above -15°C , is more difficult to model: the Rayleigh model is more reasonable for drops, as their isotopic relaxation time is large³², while the closed system model, which assumes isotopic equilibrium between all the condensed phase and the vapour, is more adequate for droplets. Indeed, an accurate model³⁴ should take into account the isotopic interactions between the water species coexisting in a cloud (vapour, cloud water, rainwater, cloud ice and graupel), the partial removal of precipitation and the variations with time of these parameters. Instead, we have chosen the two extreme hypotheses (open and closed system) by performing two parallel experiments (B and A respectively) in which one of the two assumptions is used. During evaporation, we simply assume no fractionation for solid water, because the molecular exchanges are extremely slow, and isotopic equilibrium for liquid water, which is a reasonable approximation when liquid water is mainly composed of droplets with a radius $<100\ \mu\text{m}$ (ref. 33).

The parameterization of convective clouds presently used in the LMD AGCM only provides the total change of water vapour mixing ratio at each layer without separating convective diffusion from condensation. For isotope modelling²³, however, we need to separate the two processes. We assume an intermediate step where water vapour and all isotope species are uniformly mixed throughout the vertical extension of the cloud; this assumption, associated with the cloud budget already computed, uniquely determines the vertical distribution of condensation. This seemingly excessive vertical diffusion is, in fact, compensated by intensive condensation in the upper cloud layers, associated with re-evaporation in the lower cloud layers (in this step isotopic equilibrium between liquid and vapour is assumed at the end of the process). Another necessary parameter is the fractionation coefficient at the location of phase changes. To take into account the complexity of convective diffusion, we define the fractionation coefficient as the arithmetic average of its 'initial' value (determined by the temperature at the source regions—mainly cloud base) and its final value (determined by the final temperature of the layer). This scheme was first tested on isolated clouds. It yields acceptable results but with a

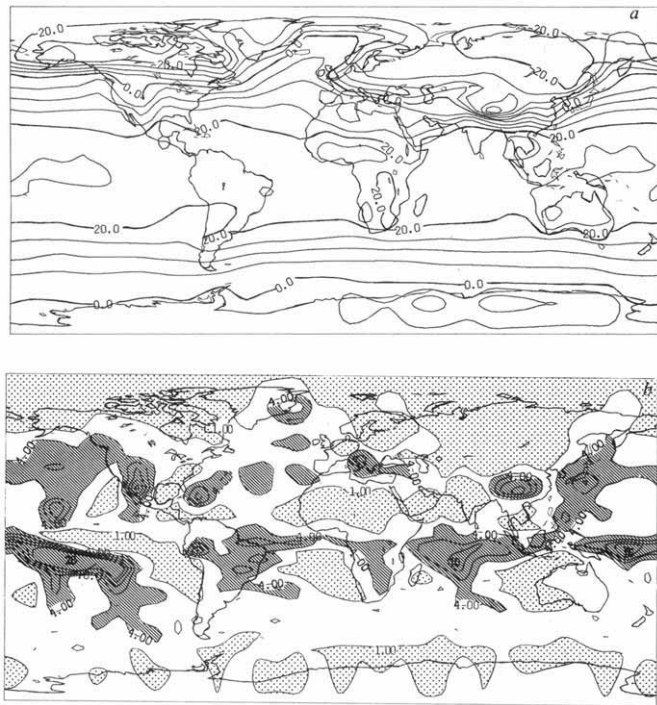


Fig. 1 Mean January simulated: *a*, surface-air temperature ($^{\circ}\text{C}$; isolines every 5°C); *b*, precipitation. Isolines every 3 mm day^{-1} up to 16 mm day^{-1} ; dotted areas, $<1\text{ mm day}^{-1}$; hatched areas, $>4\text{ mm day}^{-1}$.

tendency to systematically underestimate isotopic ratios at cloud top; this effect, however, is not too serious because it is noticeable only when clouds extend up to higher tropospheric levels where mixing ratios are, in any case, small.

Usually the supersaturation S with respect to the liquid phase is $<1\%$ in clouds³⁵ and so kinetic effects are completely negligible. However, they may become important when S is far from zero. This occurs in the case of snow crystal formation, where the supersaturation S with respect to the solid phase may reach values up to 20% (ref. 36). During re-evaporation, kinetic effects are negligible for droplets but become important for drops³², which prevail mainly in precipitating water from convective clouds. Kinetic effects at snow formation can be simply parameterized using an equivalent fractionation coefficient α_e (ref. 10) which is a decreasing function of S ; therefore, compared with equilibrium values, they tend to decrease the enrichment of falling snow and to enhance their $\delta\text{D}-\delta^{18}\text{O}$ slope. Inclusion of these effects in a dynamically simple model leads to predicted snow isotopic values in very good agreement with observed values in polar areas, both concerning the isotope-temperature and $\delta\text{D}-\delta^{18}\text{O}$ relationships¹⁰. To test the impact of kinetic effects on snow formation with our isotope modelling²³, we introduced these effects on snow formation with clouds below -15°C in only one experiment (experiment B); however, the supersaturation function used $S = 0.906 \exp(-0.008 t_c)$, t_c being the condensation temperature in $^{\circ}\text{C}$, is slightly lower than the one leading to the best agreement between observed and predicted isotopic ratios over East Antarctica¹⁰. Kinetic effects during re-evaporation of drops lead to a further enrichment of precipitating water^{37,38} and to a decrease of the $\delta\text{D}-\delta^{18}\text{O}$ slope¹. The formulation used to model these effects²³ is based on the treatment developed by Stewart³⁷ for a single drop, assuming that stationary δD and $\delta^{18}\text{O}$ values are reached at the end of re-evaporation; the enrichment increases as the relative humidity decreases. These effects have been introduced for re-evaporation of precipitating water under a convective cloud base in one experiment (B) only; the relative humidity value determining the enrichment of drops is chosen as an average of the initial and final relative humidity values of the layer.

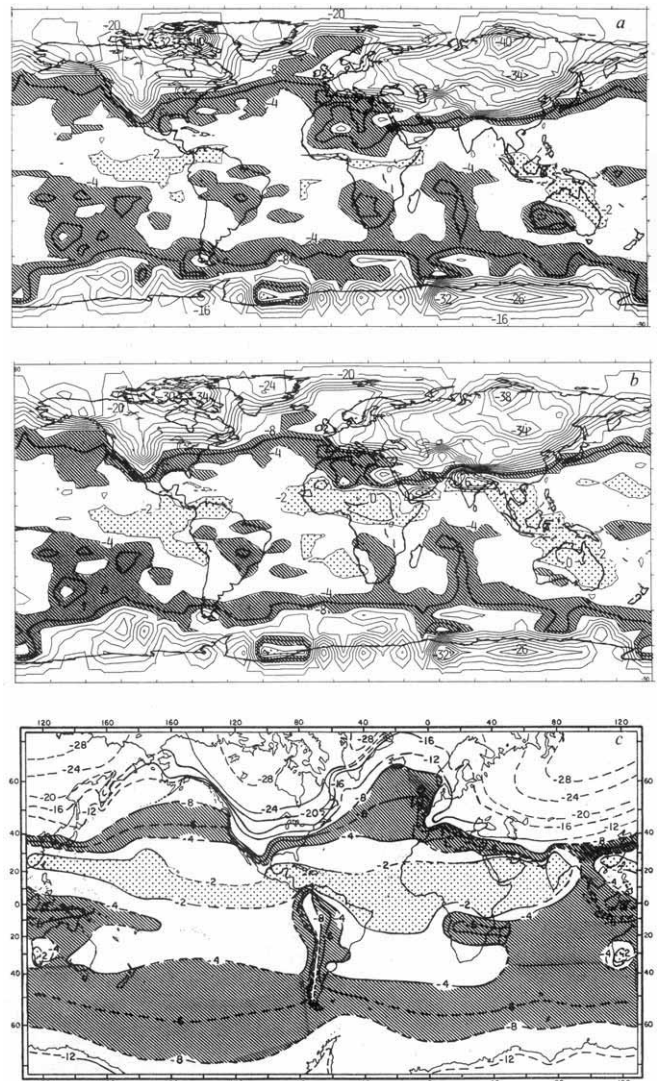


Fig. 2 Mean ^{18}O content of precipitation charts, experiments A(*a*) and B(*b*) for January to be compared with *c*, mean January observations (from J. R. Lawrence, work in preparation). Isolines every 2‰, for *c* isolines every 4‰ below -8‰ ; dotted areas, $>-2\text{‰}$; hatched areas, between -8 and -4‰ .

Numerical experiment

The preliminary tests of isotope modelling described here have been performed with a low-resolution version of the LMD AGCM: 32 points in longitude, 24 points in latitude, 11 levels, which corresponds to a horizontal mesh size of the order of $800 \times 800\text{ km}^2$ around 50° and $1,250 \times 450\text{ km}^2$ at the Equator. The numerical experiment was a simulation of the present day January climate. Initial values of atmospheric and ground variables were taken from an earlier January simulation. The initial values for vapour isotopic ratios were chosen as constant in the whole atmosphere and smaller than the observed values (-80% for H_2^{18}O and -600% for HDO , that is a deuterium excess of 40%). Surface isotope ratios were set initially at the climatological mean precipitation ratios deduced from the local surface-air temperature¹. During all the simulation, the oceanic surface-isotopic ratio was prescribed at 0% for both isotopes. All results presented here are averages over the last 20 days of this 80-day run.

The climatology of the present experiment is reasonably realistic, although it suffers from the coarse horizontal resolution. The surface-air temperature pattern (Fig. 1*a*) is well simulated, especially in the tropics³⁹. The continental gradients in the Northern Hemisphere are realistic. However, the high-latitude

temperatures in both hemispheres are markedly too warm, a defect common to all AGCMs but possibly emphasized by the low resolution used here; the particularly strong warming observed over north east Siberia is clearly associated with the low CLIMAP surface albedo value in this area. In the precipitation field (Fig. 1b), the Inter-Tropical Convergence Zone is fairly well resolved, with realistic values of 6–10 mm day⁻¹ (ref. 40), except for the Pacific branch which is underestimated and slightly shifted to the north, associated with a strongly developed South Pacific Convergence Zone with values up to 28 mm day⁻¹. These anomalous Pacific features have been noticed in other numerical experiments, using the same model; they correspond to a transient episode in the model climatology. In middle latitudes, the coarse resolution of the model yields a systematic underestimation of baroclinic instability processes and the associated low pressures and rainfall, especially in the 'roaring forties'. A related defect is an underestimation of the extent of the subtropical dry regions associated with very weak high pressures.

Concerning the isotopes, we shall mainly discuss experiment A (involving the closed-system assumption for liquid formation and no kinetic fractionation, except for evaporation at the ocean–air interface) and mention experiment B (involving the open system assumption for liquid formation and kinetic fractionation) only when major differences between A and B are observed.

Figure 2a displays the 20-day average ¹⁸O content of precipitation as simulated by experiment A. We observe a good agreement with January mean observations (Fig. 2c), with high ratios in the tropics and a decrease with increasing latitude modulated by a Northern Hemisphere planetary wave pattern. In middle to high latitudes, Figs 1a and 2a clearly show the expected positive correlation between $\delta^{18}\text{O}$ and temperature fields, low ratios being associated with low temperatures. When we look into geographical distributions in more detail, we have to be cautious because the rather large variability in space and time tends to limit the significance of 20-day averages; moreover, observations are missing in some areas (dashed contour lines on Fig. 2c). In middle latitudes, the simulated ratios are quite realistic, with values in the –8, –14‰ range in Western Europe for example. In higher latitudes, simulated ratios down to –40‰ are obtained in the coldest areas (–20, –25 °C). The accuracy of the model is not easy to verify there because observations are scarce: for instance, in Yakutsk, the simulated ratio is around –30‰ against an observed value of –34‰ (ref. 41) in December; but in Fort Smith and Edmonton, the model yields –20‰ against a range of January values from –25 to –33‰ (refs 42–47). Thus we may overestimate isotopic ratios at some locations in higher latitudes. This is especially true over ice sheet: in Greenland, our minimum simulated ratio is –22‰ whereas winter values registered in recent snow can be down to –40‰ (ref. 48); over the Antarctic Plateau, the minimum simulated value is –32‰ compared with observed values of –40 to –55‰ (refs 2, 49–51). This tendency to overestimate isotopic ratios is partially related to the unrealistic warming already observed in the model climate at high latitudes; but the values obtained over Siberia, for instance, show that the correlation between isotope and temperature biases may be disturbed by other factors such as fluctuations or biases in the origin and path of air masses. In middle to high latitudes, experiment B tends to simulate lower isotopic ratios with a difference down to –4‰, probably induced by kinetic fractionation during formation of snow crystals, as will be seen with the analysis of deuterium excess.

In the tropics, where there is little variation in temperature, the simulated $\delta^{18}\text{O}$ field exhibits a strong spatial variability, further enhanced in experiment B. These patterns are closely correlated to the precipitation field (Fig. 1b). Dansgaard¹ has already observed this 'amount effect' in tropical data sets and explained it on the one hand, by an enrichment of precipitation ratios in regions of undersaturated air or low precipitation rates, and on the other hand, by an impoverishment of water vapour in regions of intensive condensation or high precipitation rates.

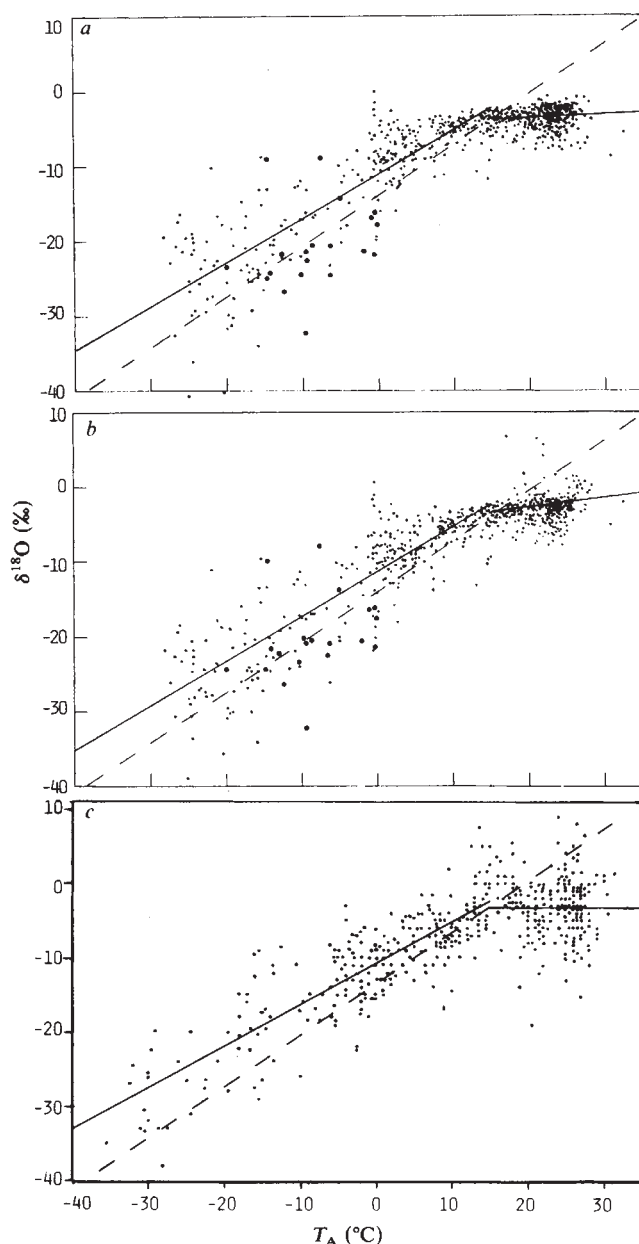


Fig. 3 Mean ¹⁸O content of precipitation as a function of mean surface-air temperature, experiments A (a) and B (b) to be compared with c, monthly mean observations for January from the IAEA network^{42–47}. Solid lines, regression lines for $T_A < 15$ °C: a, $\delta^{18}\text{O} = 0.59 T_A - 11.1$, $r = 0.86$ (correlation coefficient); b, $\delta^{18}\text{O} = 0.60 T_A - 11.5$, $r = 0.87$; c, $\delta^{18}\text{O} = 0.55 T_A - 10.7$, $r = 0.85$. For $T_A > 15$ °C: a, $\delta^{18}\text{O} = 0.05 T_A - 4.6$, $r = 0.12$; b, $\delta^{18}\text{O} = 0.13 T_A - 5.6$, $r = 0.22$; c, $\delta^{18}\text{O} = 0.006 T_A - 3.4$, $r = 0.007$. Dashed line, Dansgaard's observations¹: $\delta^{18}\text{O} = 0.69 T_A - 13.6$. Simulated Antarctica and Greenland grid point values are identified (large dots).

The first mechanism is confirmed by comparing experiments A and B: in experiment B, higher ratio regions correspond to higher enrichment by kinetic fractionation during re-evaporation under cloud base (this interpretation is corroborated by the comparative analysis of deuterium excess). Note that the closed or open system assumptions during liquid formation do not seem to have any appreciable impact on the results. More quantitatively, the simulated results are in good agreement with observations with ratios down to –4, –10‰ for high precipitation rates and up to 0–1‰ for low precipitation rates⁴¹. The best agreement is with experiment B, which corroborates the importance of kinetic fractionation during re-evaporation under cloud base: simulated ratios as high as 6‰ may seem to be overesti-

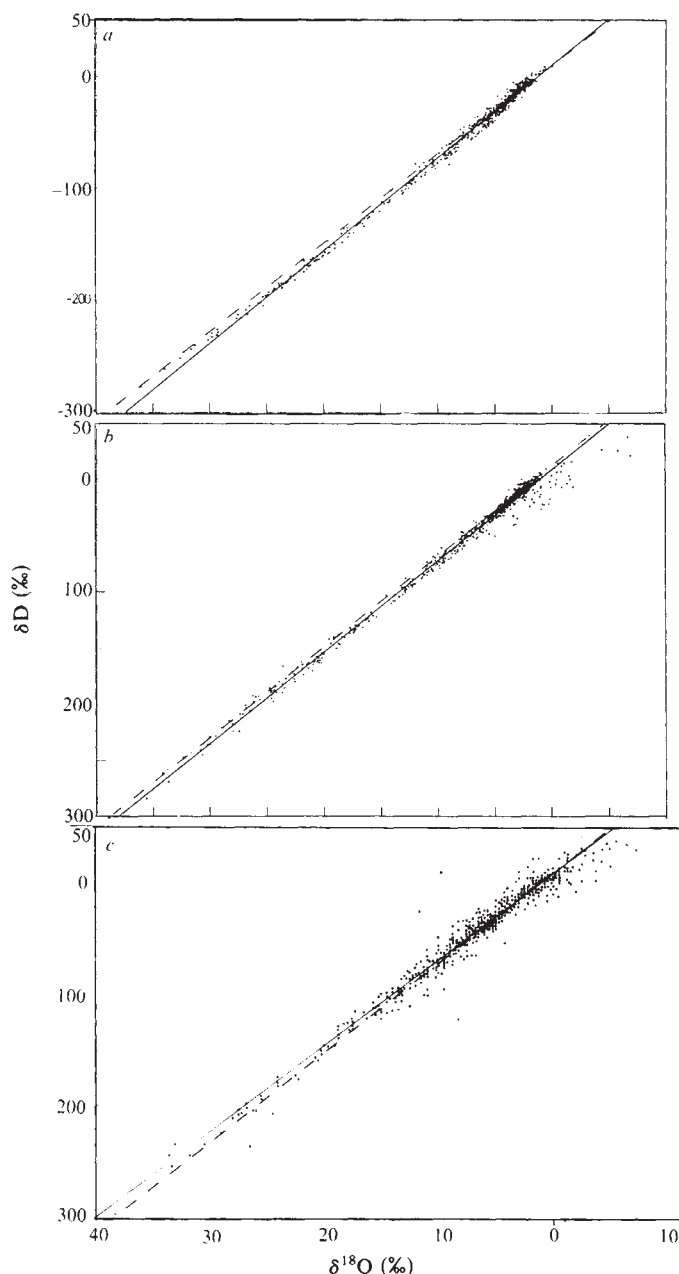


Fig. 4 Mean ^{18}O content of precipitation as a function of mean D content of precipitation, experiments A (a) and B (b) to be compared with c, monthly mean observations for January from the IAEA network⁴²⁻⁴⁷. Solid lines, regression lines: a, $\delta D = 8.3 \delta^{18}\text{O} + 11.2$, $r = 0.99$; b, $\delta D = 8.0 \delta^{18}\text{O} + 8.3$, $r = 0.99$; c, $\delta D = 7.7 \delta^{18}\text{O} + 8.5$, $r = 0.98$. Dashed line, Craig's observations⁶: $\delta D = 8 \delta^{18}\text{O} + 10$.

mated, but ratios around 8‰ are actually observed, for example, in Bamako in February or in Adelaide in January⁴¹⁻⁴⁷. Major discrepancies with observations come mainly from a displacement of some precipitation patterns in the model's climatology, for example, in central Indonesia and northern Australia, where our 20-day average 'climate' happens to be drier than normally observed and the simulated isotopic ratios are consequently higher than observed, or in the southern Pacific. Note that the dispersion in the $\delta^{18}\text{O}$ -precipitation relationship can be important; in some observations⁴¹⁻⁴⁷, or in simulated results over Australia, a positive correlation with precipitation can even occur locally. This feature probably reflects the impact of atmospheric circulation patterns on isotope contents of precipitation; simulated results over Australia are quite similar to observations in 1973, but differ from mean observations. It would be hazar-

dous, however, to explain all our isotope deficiencies by model climatic drifts. To test our isotope modelling, we need to investigate in greater detail the dependency of ^{18}O content of precipitation on both temperature and precipitation; the impact of atmospheric circulation patterns will be roughly deduced.

Figure 3a, b shows a good correlation between surface-air temperature and the 20-day average ^{18}O content of precipitation simulated in experiments A and B respectively; Fig. 3c displays the corresponding monthly average observed field for January, extracted from IAEA data over the 1953-75 period⁴²⁻⁴⁷. At temperatures $< 15^\circ\text{C}$, the correlation is quite significant in all of Fig. 3, with correlation coefficients around 0.85. The simulated and observed regression lines and dispersions are also quite similar. The differences noted in the geographical charts between experiments A and B do not affect the $\delta^{18}\text{O}$ -temperature relationship. This seems to contradict earlier conclusions obtained from simple models^{10,32}, which do not, however, include dispersion effects such as the origin and path of air masses; the reason could also be that the model produces temperatures that are too warm and a relatively large proportion of convective precipitation in polar areas, thus underestimating the importance of kinetic effects during formation of snow crystals, which are taken into account in non-convective systems only and for snow formed below -15°C . Dansgaard's¹ linear relation between $\delta^{18}\text{O}$ and temperature below 15°C has also been plotted in Fig. 3. We observe that both IAEA-observed and simulated ratios are, on average, higher than Dansgaard's values, with an increasing discrepancy in colder regions. Dansgaard's data are more reliable at high latitudes where IAEA stations are scarce; the low values he obtained there have been confirmed by more recent studies^{2,52}. Note that all ice cap data to which we refer^{1,2,48,52} concern annual means; for Antarctica, the displacement could be further enhanced when considering January values^{49,50}. These relatively low values over polar regions (Antarctica and Greenland) associated with an enhanced dependency on temperature could be due to (1) increased distance from water isotope source regions, inducing a larger amount of fractionation processes, and (2) altitude, which causes precipitations to originate from higher tropospheric levels. Such a systematic polar cap effect is actually simulated by the model as can be seen by the large dots on Fig. 3a, b, corresponding to Antarctica and Greenland grid point values; these values are indeed close to ice cap data⁴⁸. It is, however, difficult to assess the efficiency of our isotope modelling in these regions as temperatures there are systematically too high.

At temperatures above 15°C , both correlation and dependency on temperature totally disappear in observed data and decrease strongly in our simulation. The weakening of the temperature-isotope relation is mainly due to the predominance of convective systems, accompanied by intense mixing of water originating from different layers. The dispersion (further enhanced in experiment B by kinetic effects under cloud base) mainly reflects dependency on precipitation amount. The general dependency of $\delta^{18}\text{O}$ on precipitation for temperatures higher than 15°C clearly appears on both experiments with correlation coefficients around -0.4 and simulated regression lines: $\delta^{18}\text{O} = -0.50P - 2.8$ for experiment A and $\delta^{18}\text{O} = -0.79P - 2.0$ for experiment B, with $\delta^{18}\text{O}$ in ‰ and precipitations P in 100 mm per month. The results of experiment B are in particularly good agreement with January mean observations: $\delta^{18}\text{O} = -0.93P - 1.8$ with a similar correlation coefficient. This again pinpoints the importance of kinetic fractionation during re-evaporation under cloud base.

Deuterium content of precipitation has similar dependencies on temperature and precipitation; therefore, at this stage, we just need to study its relation with ^{18}O content of precipitation (Fig. 4a, b, c). Correlation is high in all of Fig. 4, with correlation coefficients around 0.98. The simulated ratios are in close agreement with observations and their dispersion around Craig's meteoric water line⁶ $d = \delta D - 8\delta^{18}\text{O} = 10\text{‰}$ is similar. In the high ratio range, the observed decrease in slope and increase in

dispersion (Fig. 4c) is well simulated only by experiment B: this supports the importance of kinetic fractionation during re-evaporation under cloud base¹. In the low ratio range, experiment B with kinetic fractionation during snow crystal formation shows a better agreement with observations, with a slightly greater $\delta D-\delta^{18}O$ slope than in experiment A. Although they have a smaller amplitude, these features corroborate simple model results¹⁰.

Conclusion

We have presented the first simulation of the atmospheric cycles of HDO and $H_2^{18}O$. Many features of our simulations are in good agreement with real conditions. Most of the differences we have observed on geographical charts seem mainly due to

local differences between simulated and observed climate; this is confirmed by the fact that the $\delta^{18}O$ -temperature, $\delta^{18}O$ -precipitation and $\delta D-\delta^{18}O$ relations are realistically simulated. The use of higher resolution in future experiments will improve the climatology of the model and, therefore, many aspects of the isotope simulations. The model in its present form looks accurate enough to be used for palaeoclimatic reconstructions.

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Molecular cloning of a new transforming gene from a chemically transformed human cell line

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Molecular cloning of the transforming gene from a chemically transformed human osteosarcoma-derived cell line enables the gene to be mapped to chromosome 7 (7p11.4-7qter) and by this criterion and by direct hybridization to be shown to be unrelated to known oncogenes.

DOMINANT transforming genes have been detected in several different types of chemically induced tumours and in cells transformed *in vitro* by chemical carcinogens. Many of the transforming genes identified in these studies were members of the *ras* family of oncogenes. Cellular homologues of the oncogene of Harvey sarcoma virus (*ras^H*) were activated in chemically induced squamous cell carcinomas and papillomas in mice^{1,2}, chemically induced mammary tumours in rats³ and guinea pig

cells transformed *in vitro* by chemical carcinogens⁴. Similarly, cellular homologues of the oncogene of Kirsten sarcoma virus (*ras^K*) were activated in 3-methylcholanthrene-induced mouse sarcomas⁵ and in mouse fibroblasts⁶ transformed *in vitro* by chemical carcinogens. A third member of the *ras* gene family (*ras^N*) was activated in chemically induced thymic lymphomas of mice⁷. Studies of these systems should reveal the mechanisms of activation of *ras* genes by classes of chemical carcinogens

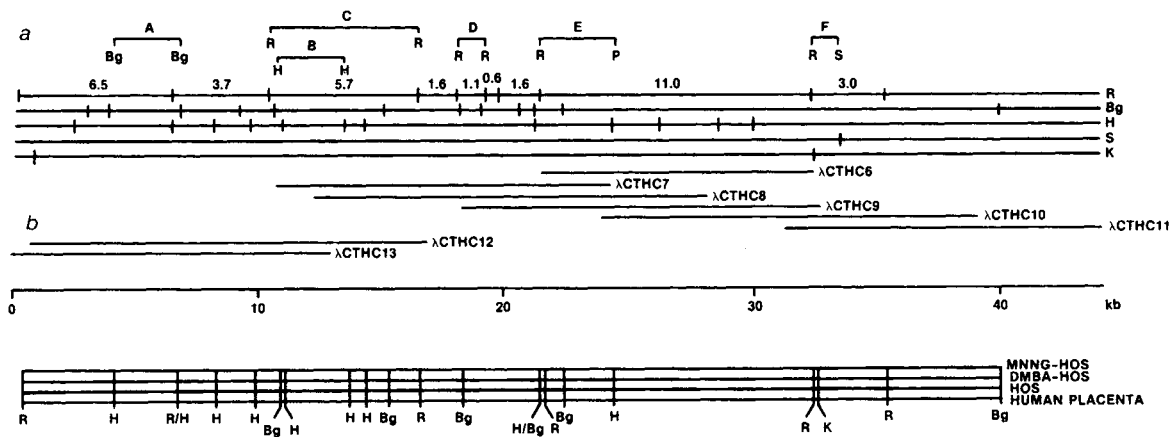


Fig. 1 *a*, Transforming DNA sequences of MNNG-HOS cells. An 11.0-kb human DNA fragment present in *EcoRI*-digested DNA from a MNNG-HOS secondary transfectant (λ CTHC6) was cloned from a library constructed in the λ gtWES cloning vector. Phage libraries were constructed by the method of Hohn and Murray⁴⁹. The library was screened, without prior amplification, by the method of Benton and Davis⁵⁰ using *Blu*8 plasmid DNA¹⁸ which was labelled with ³²P by nick-translation⁵¹ as a probe. The 11.0-kb *EcoRI* fragment was subcloned into pBR322 and the subclone was used as a probe to isolate recombinant DNA clones (λ CTHC7-11) that contained adjacent DNA sequences from a second library. The latter was constructed by first partially digesting DNA from an MNNG-HOS transfectant with *Sau*3A and then introducing the partially digested DNA into the EMBL3 cloning vector⁵². Clones λ CTHC-12 and -13 were isolated from the same EMBL3 library using a probe prepared from a restriction fragment (fragment B) produced by digesting λ CTHC7 with *Hind*III. A restriction map of the cloned cellular DNA is presented. Six specific restriction fragments (fragments A-F) were subcloned into pBR322. The sizes in kilobases of the *EcoRI* fragments are indicated. *b*, Partial restriction maps of the gene present in human placenta, MNNG-HOS cells, DMBA-HOS cells and HOS cells. The maps were determined from the results of hybridization analysis in which restriction fragments A-F (a) were used to detect related sequences in human DNAs that had been digested either with *EcoRI*, *Hind*III and *Bgl*II or with *Kpn*I and *Bgl*II. DNAs were prepared exactly as described previously¹⁶, digested with three to fivefold excess of restriction enzyme, electrophoresed in agarose (1% w/v) gels and blotted onto nitrocellulose as described by Southern¹⁷. Filters were hybridized to DNA fragments labelled with ³²P by nick translation⁵¹, in stringent conditions (50% formamide, 5 $\times$ SSC, 42 $^{\circ}$ C) for 24 h and washed with a final stringency of 0.2 $\times$ SSC at 68 $^{\circ}$ C. Restriction endonucleases used were *EcoRI* (R), *Bgl*II (Bg), *Hind*III (H), *Sal*I (S), *Kpn*I (K) and *Pst*I (P).

which, as environmental and dietary contaminants, are suspected of contributing to the incidence of cancer in man⁸⁻¹⁰.

In contrast to the abundance of studies on chemically transformed rodent cells, there are only a few reported examples of human cells transformed by direct treatment with chemical carcinogens¹¹⁻¹⁴. The transformation of human fibroblasts¹¹ and human osteosarcoma (HOS)^{12,13} cells by chemical carcinogens *in vitro* has been reported and in initial studies we examined the ability of DNA prepared from these chemically transformed human cell lines to transform NIH 3T3 cells^{15,16}. Our results showed that DNA from a HOS cell line that had been chemically transformed *in vitro* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)¹² could transform NIH 3T3 cells. In contrast, DNA from 7,12-dimethylbenz(a)anthracene (DMBA) transformed HOS cells¹³, parental HOS cells or 4-nitroquinoline-1-oxide transformed human fibroblasts (HUT14) did not transform NIH 3T3 cells¹⁵, suggesting that MNNG may have caused genetic alterations in HOS cells that gave rise to a transferable transforming gene. Recently, we have demonstrated that this transforming gene is not a member of the *ras* family of genes¹⁶, and we now report the molecular cloning of the MNNG-HOS transforming gene, its comparison with known oncogenes and its human chromosomal localization.

Molecular cloning

High molecular weight DNA from MNNG-HOS cells can efficiently transform mouse NIH 3T3 cells, and DNA from the transformed NIH 3T3 cells (primary transfectants) can be used in a second round of transfection to induce secondary transfectants^{15,16}. Southern analysis¹⁷ of *EcoRI*-digested transfectant DNA with a probe specific for human highly repeated *Alu* DNA sequences (Blu8)¹⁸ showed that the secondary transfectants contained conserved human *EcoRI* DNA fragments of 11.0, 5.7 and 3.7 kilobases (kb). This observation suggested that these three human DNA fragments are closely associated with the transforming gene. In our strategy for the molecular cloning of the MNNG-HOS transforming gene, we first used the probe specific for human highly repeated DNA sequences to isolate

the 11.0-kb *EcoRI* DNA fragment. The 11.0-kb human DNA fragment present in *EcoRI*-digested DNA from an MNNG-HOS secondary transfectant was enriched by centrifugation through a sucrose gradient and cloned into the EK2 vector λ gtWES. From a library of 400,000 recombinant phage, we obtained one isolate (λ CTHC-6, Fig. 1a) that possessed human repeated DNA sequences and contained an 11.0-kb *EcoRI* DNA fragment.

Regions of the transforming gene which were adjacent to the 11.0-kb *EcoRI* fragment were isolated from a library obtained using DNA from an MNNG-HOS secondary transfectant. The DNA was first partially digested with *Sau*3A and then cloned into the EMBL3 cloning vector. A library of 2 $\times$ 10⁶ recombinant phage was amplified and phage that contained regions of the transforming gene immediately adjacent to the 11.0-kb *EcoRI* fragment were isolated from portions of the amplified library using the cloned 11.0-kb *EcoRI* human DNA fragment as a probe. More distant regions of the transforming gene were isolated in genomic library 'walking' experiments (Fig. 1a). Figure 1a shows restriction map of 44 kb of cellular DNA present in seven recombinant clones isolated from the library.

To determine which parts of the cloned DNA represented the MNNG-HOS transforming gene, the λ clones (λ CTHC6-13) and specific restriction fragments (fragments A-F, Fig. 1a) were used as probes in Southern analyses¹⁷ to detect related sequences in DNA from MNNG-HOS secondary transfectants. The results of these analyses indicated that 26 kb of DNA which includes *EcoRI* human DNA fragments of 11.0, 5.7, 3.7, two 1.6, 1.1 and 0.6 kb (Fig. 1a) is part of the transforming gene, as these DNA sequences are invariably present in DNA from MNNG-HOS secondary transfectants. For example, probes prepared from the cloned 11.0-kb *EcoRI* fragment present in λ CTHC6 and from fragment D (Fig. 1a) both detected an 11.0-kb DNA fragment in *EcoRI*-digested genomic DNA from secondary transfectants. In contrast, the 3.0- and 6.5-kb *EcoRI* fragments that occur respectively at the right and left ends of the cloned DNA (Fig. 1a) were not detected in all of the secondary transfectants. Thus, the data presented in Fig. 3 show that a probe prepared from λ CTHC10 only detected the 3.0-kb *EcoRI* DNA fragment in

two of the four secondary transfectants. In addition, the results of similar studies in which λ CTHC13 was used as a probe showed that only 7 of 10 secondary transfectants examined contained the 6.5-kb DNA fragment (data not shown). When taken together, these observations indicate that the 6.5- and 3.0-kb *EcoRI* DNA fragments contain flanking DNA sequences that can be removed during transfection without affecting the activity of the MNNG-HOS transforming gene.

In transfection experiments, when examined individually, none of the λ clones (λ CTHC6-13) could transform NIH 3T3 cells. However, this result was not unexpected as each clone contains only part of the transforming gene. Examination of the biological activity of the cloned MNNG-HOS transforming gene must await the reconstruction of the intact gene.

Arrangement of the MNNG-HOS gene

To determine whether the transforming gene was rearranged in MNNG-HOS cells, portions of the cloned gene (Fig. 1a, fragments A-F) were used as probes to detect related sequences in DNA prepared from several different human cell lines and from human placenta. A probe prepared from an *EcoRI*-*PstI* restriction fragment (fragment E, Fig. 1a) hybridized to an 11.0-kb DNA fragment in *EcoRI*-digested human placental DNA (Fig. 2B, lane h), as well as DNA prepared from other human cell lines (Fig. 2B, lanes f, g, i-m). Data obtained in this experiment and in other Southern analyses of DNA digested either with *EcoRI*, *HindIII*, *BglII* and *KpnI* or with *KpnI* and *BglII* allowed us to construct partial restriction maps of the related DNA sequences present in human placenta, MNNG-HOS cells, DMBA-HOS cells and HOS cells. The partial restriction maps obtained for all four human DNAs are identical and are shown in Fig. 1b. Thus, at this level of resolution there are no detectable rearrangements of these DNA sequences in the MNNG-HOS cell line.

Relationship to oncogenes and location

We reported previously that the MNNG-HOS transforming gene is not a member of the *ras* family of oncogenes¹⁶. Hybridization of the cloned MNNG-HOS transforming gene to cloned viral and cellular oncogenes confirmed this result and also showed that the transforming gene is not homologous by DNA hybridization to *mos*, *myc*, *myb*, *src*, *sis*, *rel* and *Blym* (data not shown). Furthermore, the restriction map of the MNNG-HOS transforming gene is not the same as those of *ras*^N (refs 19, 20) and *Blym* (ref. 21) or of the human homologues of *ras*^H (ref. 22), *ras*^K (ref. 19), *mos* (ref. 23), *myc* (ref. 24), *abl* (ref. 25), *sis* (ref. 26) and *fes* (ref. 27).

As a further characterization of the MNNG-HOS gene, we determined its human chromosomal location. Southern analysis of DNA from 12 different rodent-human somatic cell hybrids containing unique complements of human chromosomes (Table 1) was used to determine the chromosomal location of the MNNG-HOS transforming gene. Cell hybrids were characterized by karyotype analysis and by expression of isozyme markers that have been previously assigned to each of the human chromosomes²⁸⁻³¹. DNA prepared from each cell hybrid was digested with *HindIII* and subjected to Southern analysis using the *EcoRI*-*PstI* DNA fragment (fragment E, Fig. 1a) as a probe. In stringent conditions of hybridization, probe E detects a single 3.2-kb human *HindIII* restriction fragment (Fig. 4A, lanes g, i) but does not hybridize to rodent DNA (Fig. 4A, lane h). Figure 4 gives examples of these analyses while Table 1 shows the human chromosome complement of the hybrid cell lines and the results obtained when the DNAs from these hybrids were scored for the presence of the 3.2-kb *HindIII* restriction fragment. The results are consistent only with assignment of the MNNG-HOS transforming gene to chromosome 7. To refine this initial result, we have analysed a human-mouse hybrid cell line (53-87-3 c136 is c12 sub7) which retains only the portion of human chromosome 7 from 7p11.4 to 7qter (ref. 30) and has

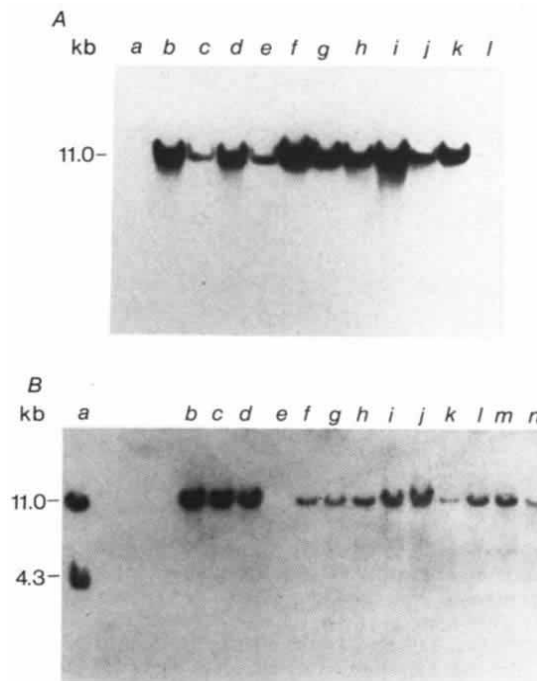


Fig. 2 Hybridization of human DNA and DNA from MNNG-HOS secondary transfectants to cloned DNA sequences. **A**, 10 μ g of each DNA (lane a, NIH 3T3; lanes b-l, independent MNNG-HOS secondary transfectants) was digested with *EcoRI*, subjected to electrophoresis in agarose gels (0.8 w/v) and transferred to a nitrocellulose filter as described by Southern¹⁷. The filters were hybridized (50% formamide, 5 $\times$ SSC, 42 $^{\circ}$ C) for 21 h to a ³²P-labelled probe that was prepared from the 11.0-kb *EcoRI* restriction fragment present in clone λ CTHC6 (Fig. 1a) by nick translation⁵¹. The 11.0-kb *EcoRI* restriction fragment was first subcloned into pBR322. The plasmid DNA was digested with *EcoRI* and the 11.0-kb restriction fragment was purified by electrophoresis in agarose gels. **B**, 10 μ g of each DNA (lane a, NIH 3T3 DNA containing 5 genomic equivalents of the 11.0-kb *EcoRI* restriction fragment cloned into pBR322; lanes b-d, independent MNNG-HOS secondary transfectants; lane e, NIH 3T3; lane f, DMBA-HOS¹⁶; lane g, MNNG-HOS¹⁶; lane h, HOS¹⁶; lane i, human placenta; lane j, human fibrosarcoma HT1080 (ref. 15); lane k, human teratocarcinoma, PA1 (ref. 16); lane l, human nasopharyngeal carcinoma; lane m, Wilms' tumour; lane n, human fibrosarcoma NF-1 (ref. 16)) was digested with *EcoRI* and subjected to Southern analysis as described in **A**. The nitrocellulose filters were hybridized to a ³²P-labelled probe prepared from an *EcoRI*-*PstI* restriction fragment (fragment E, Fig. 1a).

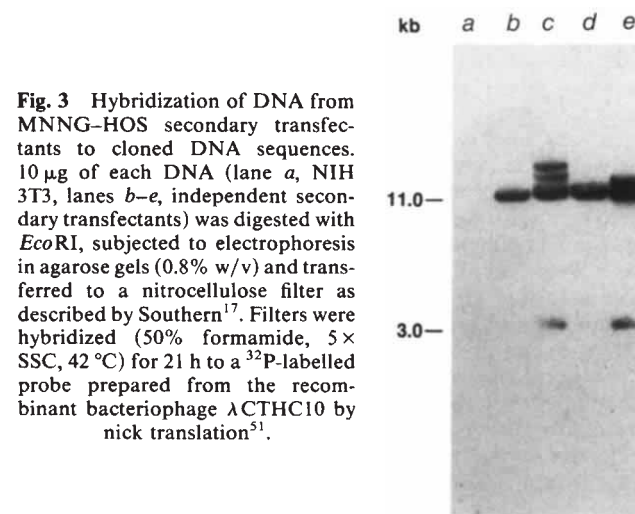


Fig. 3 Hybridization of DNA from MNNG-HOS secondary transfectants to cloned DNA sequences. 10 μ g of each DNA (lane a, NIH 3T3; lanes b-e, independent secondary transfectants) was digested with *EcoRI*, subjected to electrophoresis in agarose gels (0.8% w/v) and transferred to a nitrocellulose filter as described by Southern¹⁷. Filters were hybridized (50% formamide, 5 $\times$ SSC, 42 $^{\circ}$ C) for 21 h to a ³²P-labelled probe prepared from the recombinant bacteriophage λ CTHC10 by nick translation⁵¹.

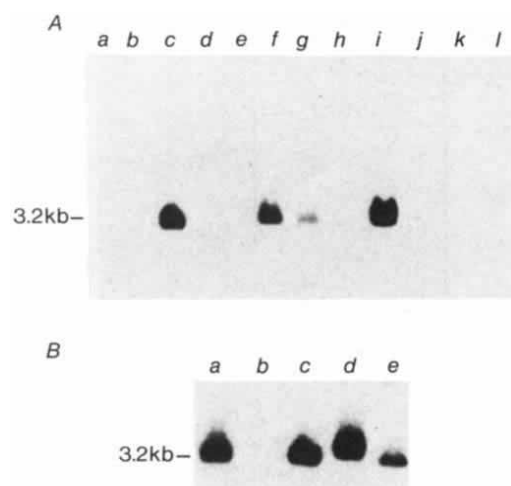


Fig. 4 Hybridization of a probe specific for the MNNG-HOS transforming gene to DNA from rodent-human cell hybrids. **A**, Segregation of the MNNG-HOS transforming gene in rodent-human hybrids. High molecular weight DNA (10 µg) isolated from 77-B10 c126 (lane a), DSK 1B2A5 c120 (lane b), Nude 9 (lane c), PAF × BALB IV c15 (lane d), M44 c12S5 (lane e), 53-87-1 c121 (lane f), HL60 human promyelocytic leukaemia cell line (lane g), F9 mouse strain 129 teratocarcinoma cell line (lane h), JD-38 human Burkitt lymphoma cell line (lane i), 706B6-40 c117 (lane j), 77 B10 c18 (lane k) and 77 B10 c133 (lane l) cell lines was digested with a fivefold excess of restriction endonuclease *Hind*III, subjected to electrophoresis in 1% agarose gels, then blotted onto nitrocellulose filters. Filters were hybridized in stringent conditions, (50% formamide, 5 × SSC, 10% dextran sulphate at 42 °C) for 20 h with ³²P-labelled, nick-translated fragment E (Fig. 1a). Hybridized blots were exposed to Kodak XAR-5 film at -70 °C in the presence of Kodak intensifier screens for 18 h. **B**, High molecular weight DNA (10 µg) isolated from Ag876II human Burkitt's lymphoma cell line (lane a), NP3, BALB/c mouse myeloma cell line (lane b), Nude 9 (lane c), 53-87-1 c121 (lane d), 53-87-3 c136 is c12 sub7 (lane e) cell lines was digested with a fivefold excess of *Hind*III and subjected to Southern analysis¹⁷ as described above. The rodent-human cell lines used in these experiments have been described previously²⁸⁻³¹.

lost the distal part of the short arm. This analysis (Fig. 4B) shows that hybrids containing only human chromosome 7 (Fig. 4B, lane d) or only 7p11.4 to 7qter (Fig. 4B, lane e) have retained the MNNG-HOS transforming gene. Thus, this gene is located on chromosome 7 between 7p11.4 and 7qter.

The localization of the MNNG-HOS transforming gene on chromosome 7 provides additional evidence that this gene is not a human homologue of *ras*^H, *ras*^K, *fos*, *mos*, *myb*, *myc*, *raf*, *abl*, *fes*, *src* or *sis*, and does not represent an activated human *ras*^N, *Blym* or *myc*^N locus because these human proto-oncogenes are located on different human chromosomes (reviewed in ref. 32). The human homologue of the avian erythroblastosis virus (AEV) transforming gene (*erbB*) has been assigned to chromosome 7 (7pter-7q22)³³. Because the MNNG-HOS transforming gene is located between 7p11.4 and 7qter, chromosomal assignment could not exclude the possibility that it is related to *erbB*. However, the MNNG-HOS transforming gene failed to hybridize to probes prepared from the *erbB* portion of AEV (the 2.5-kb *Pvu*II-*Pvu*II fragment described in ref. 34) or from part of the human homologue of *erbB* (the 2.5-kb *Hind*III-*Eco*RI fragment he-B, described in ref. 35; data not shown). These data demonstrate that the MNNG-HOS transforming gene is not directly homologous to *erbB*. However, the cloned he-B and viral *erbB* DNA sequences only represent parts of the cellular gene that show homology to *erbB* (ref. 35) and we cannot exclude the possibility that the MNNG-HOS transforming gene may be related to other parts of this cellular gene.

Assuming that the MNNG-HOS transforming gene is unrelated to other transforming genes, we suggest that the three-letter abbreviation, *met*, should be used when referring to this gene.

Table 1 Detection of the MNNG-HOS transforming gene in rodent-human hybrid cells

Hybrid	Human Chromosomes																						Human met Gene	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		X
DSK 1B2A5 c12 Suba																								-
DSK 1B2A5 c120																								-
Nu 9																								+
PAF x Balb IV c15																								+
706B6-40 c117																								-
77-B10 c18																								-
77-B10 c112																								-
77-B10 c126																								-
77-B10 c133																								-
GM x LM c13a																								+
GM x LM c16b																								+
M44 c12S5																								+
53-87-1 c121																								+
53-87-3 c136 is c12 sub 7																								+

The rodent-human hybrid cells have been described previously and the presence of particular human chromosomes was determined by chromosomal and isoenzyme analyses²⁸⁻³¹. The presence of the transforming gene in each hybrid cell line was determined by Southern analysis¹⁷ as described in Fig. 4 legend. The black squares indicate the presence of a particular human chromosome. Hybrid cell line M44 c12S5 contains only a 14q+ t(8;14) human chromosome⁴⁷ and hybrid cell line 52-87-3 c136 is c12 sub7 contains only a portion of a human chromosome 7, from 7p11.4 to 7qter (ref. 30).

Discussion

This new transforming gene has been mapped to human chromosome 7 (7p11.4 to 7qter) and by these criteria or by direct hybridization seems to be unrelated to other known oncogenes.

The presence of *met* on chromosome 7 is of interest because several different types of human neoplasia are associated with alterations in chromosome 7. Monosomy of chromosome 7, alone or in combination with other chromosomal abnormalities in the same cell, is one of the nonrandom aberrations seen in most subtypes of acute non-lymphocytic leukaemia³⁶⁻³⁸. Several types of rearrangements involving chromosome 7 have also been detected in cells from individuals with ataxia telangiectasia. These include both translocations [t(7;7) and t(7;p14)] and chromosomal inversions³⁹. A more detailed localization of the position of the *met* transforming gene on chromosome 7 by *in situ* hybridization may allow a better assessment of the possible involvement of this transforming gene in specific disorders.

The activation of a non-*ras* transforming gene in MNNG-HOS cells is at variance with the observations that most of the transforming genes activated in chemically transformed rodent cells are members of the *ras* family of oncogenes¹⁻⁷, and that activated *ras* genes are detected in a variety of human neoplasias, such as lung and colon tumours⁴⁰⁻⁴², which are believed, at least in part, to result from exposure to environmental carcinogens. Because we have been able to examine only a limited number of human cell lines, it is not yet possible to determine whether the activation of non-*ras* transforming genes is a consistent feature of human cells that are transformed *in vitro* by chemical carcinogens or whether the HOS cell line possesses a unique predisposition to activation of the *met* transforming gene. Some of the transforming genes detected in chemically transformed rodent cells were, however, also unrelated to *ras*. These include genes from DMBA-induced transplantable mammary tumours⁴³ and mineral oil induced B- and T-cell neoplasms⁴⁴.

No detectable rearrangements were observed in the restriction map comparison of the *met* gene in genomic DNA of MNNG-HOS with other human DNA samples. Activation of the transforming gene might therefore involve more subtle changes in the DNA such as the activation of the human *ras*^H gene in the EJ and T24 bladder carcinoma cell lines which involves a single G → T point mutation⁴⁵⁻⁴⁷. It is conceivable that a point mutation is also involved in the activation of the MNNG-HOS transforming gene. Alternatively, there may be small insertions, deletions or rearrangements that were not detected in these analyses. As DNA, RNA and proteins can be easily prepared from MNNG-

HOS cells and as the chemistry of the interaction of simple methylating carcinogens such as MNNG is particularly well defined⁴⁸, a comparison of the *met* gene from MNNG-HOS cells with the parental HOS cell gene may provide a model system with which to study the mechanisms of transformation of human cells by this carcinogen.

In at least one *N*-nitroso-*N*-methylurea (NMU) induced rat mammary tumour, activation of the *ras*^H transforming gene involves a simple G→A point mutation³. Notably, this is precisely the mutation that NMU would be predicted to induce if it reacted directly with the transforming gene. It will be interesting to see whether the precise method of activation of this transforming gene and of other transforming genes detected in chemically transformed cells also reflects the particular

chemicals used to induce transformation. Further characterization of the *met* transforming gene and its comparison with the non-transforming allele may provide an interesting insight into one of the ways in which chemical carcinogens can activate proto-oncogenes in human cells.

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Sequence requirements for nuclear location of simian virus 40 large-T antigen

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A point mutation in the simian virus 40 large-T gene, which was generated by mixed oligonucleotide mutagenesis and resulted in the conversion of Lys 128 to Thr, produced a large-T antigen that was detected in the cytoplasm but not the nucleus of cells. Deletions within the surrounding sequence Lys-¹²⁸Lys-Lys-Arg-Lys-Val-Glu also produce cytoplasmic large-T and define a region of the protein involved in nuclear location.

THE large-T antigen of simian virus 40 (SV40), molecular weight *M*_r 94,000, is sufficient to transform established and non-established rodent cells *in vitro* and to induce tumours in newborn hamsters¹. It is also required for the productive infection of simian cells by SV40².

Large-T is a predominantly nuclear protein³ but is also found in a modified form in the plasma membrane^{4,5}. During a productive infection the nuclear form of large-T regulates the level of viral gene expression and stimulates viral DNA replication through an interaction with the viral origin of DNA replication^{6–8}. To investigate whether DNA binding by large-T is also involved in transformation we have introduced mutations⁹ into a region coding for a putative DNA binding domain of the

protein^{6,10–13}. Within this domain is a striking tract of five basic amino acids (¹²⁷Lys-Lys-Lys-Arg-Lys¹³¹) just downstream from a cluster of serine and threonine phosphorylation sites¹⁴. As the sense of the codons for lysine is unaffected by the action of the commonly used mutagen, sodium bisulphite, to mutate this region we devised a modification of oligonucleotide-directed mutagenesis^{15,16} that allows the introduction of several defined point mutations in a single experiment.

The large-T protein encoded by one of the mutants generated in this manner contains a threonine residue in place of Lys 128. This mutant large-T, although able to transform Rat-1 cells with wild-type efficiency as measured by focus formation in high serum, accumulates in the cytoplasm rather than the nucleus of

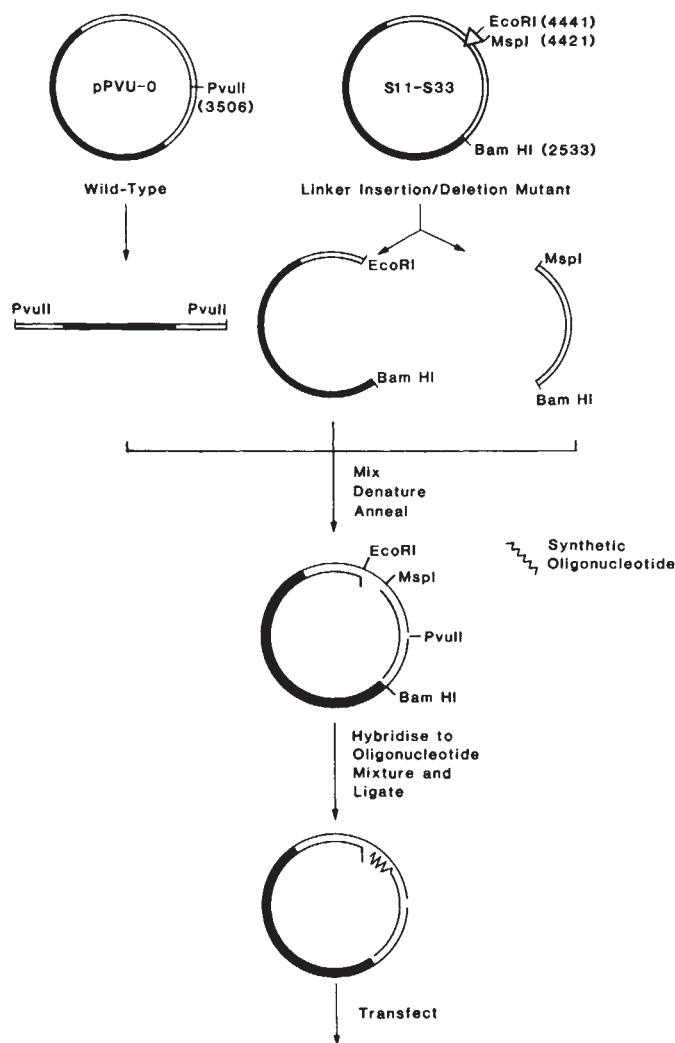


Fig. 1 Mixed oligonucleotide mutagenesis. Plasmid pPVU-0 (ref. 23) contains the wild-type SV40 early region from *Bam*HI (2,533) to *Pvu*II (270) between the *Bam*HI (375) and *Pvu*II (4,676) sites of pBR328 (ref. 37) but with a deletion of 3 base-pairs at the junction of the *Pvu*II sites, thus destroying the enzyme recognition site at this point. In the figure, plasmid DNA sequences are shaded whereas the SV40-derived sequences are not. S11-S33 (ref. 9) is a derivative of pPVU-0 that contains a 10 bp *Eco*RI linker (CGGAATTCCG) spanning a deletion between SV40 nucleotides 4441 and 4421. An *Msp*I site is created at the junction of linker and SV40 nucleotide sequences around position 4421. *Msp*I-*Bam*HI and *Bam*HI-*Eco*RI restriction fragments from S11-S33 were purified by agarose gel electrophoresis followed by phenol extraction⁹ and were mixed in equimolar proportions with pPVU-0 DNA linearized at its unique *Pvu*II site. After denaturation with alkali and subsequent renaturation²³ about 10–20% of the products migrated as form II (open duplex circles) during agarose gel electrophoresis and were presumed to be circular heteroduplexes of the form shown above. The hybridization products (2.5 µg DNA) were dissolved, after ethanol precipitation in 10 µl of ligation buffer (50 mM Tris-HCl pH 8.0, 7.5 mM MgCl₂, 1 mM ATP, 10 mM dithiothreitol (DTT) and 0.02% gelatin) and to this was added 2.5 µg of the oligonucleotide mixture (synthesized as in ref. 38) and phosphorylated by T4 polynucleotide kinase, also in 10 µl of ligation buffer. The components were mixed initially at 60 °C, the temperature was then gradually reduced to 0 °C over a 6 h period, after which additional dithiothreitol (to 5 mM) and T4 DNA ligase (6 units) were added. After 1 h at 0 °C, ligation was continued for 4 h at 15 °C. Some of the ligated material was then used directly for transfection. Alternatively, after phenol extraction and ethanol precipitation aliquots were treated with S₁ nuclease (50 units per 10 µl/0.4 µg DNA for 20 min at 37 °C) before neutralization and transfection. S₁ nuclease treatment reduced the number of transfectants about 10-fold. Parallel experiments showed that significant cleavage of nicked circular plasmid DNA did not occur at the S₁ nuclease concentration used.

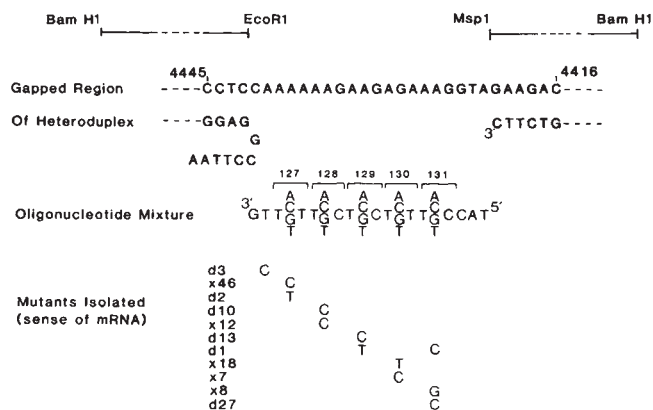


Fig. 2 Mutants obtained using mixed oligonucleotide mutagenesis. The composition of the synthetic oligonucleotide mixture is shown above together with the single-stranded region of the gapped heteroduplex, formed as in Fig. 1, to which the oligonucleotides were hybridized. Note that after hybridization the synthetic oligonucleotides are exactly apposed at their 5' ends to the double-stranded region of the heteroduplex and can therefore be ligated directly into the heteroduplex. The nucleotide sequence alterations in mutant plasmids recovered from the procedure described in Fig. 1 are shown. The inferred alterations to the amino acid sequence of large-T are presented in Table 1. The mutation found in plasmid d3 should not theoretically have been introduced by the oligonucleotide mixture used. It was not studied further because it does not alter the amino acid sequence of large-T.

the transformed cells. The phenotypes of other mutants with lesions in the same area help to define a region around Lys 128 which is required for the nuclear localization of large-T.

Mixed oligonucleotide mutagenesis

Oligonucleotide-directed mutagenesis is currently the method of choice for the introduction of single, precisely defined mutations. However, this method is not generally used for the creation of large families of point mutants because of the labour or expense involved in obtaining a large number of individual synthetic oligonucleotides. In the procedure described here a mixture of oligonucleotides synthesized on a single resin was used as the mutagen.

The mixture of oligonucleotides, each of 20 residues, was synthesized by including an equimolar mixture of all four nucleotides at five selected positions along its length (Fig. 2). The degenerate positions correspond to the second nucleotides in the codons for the basic sequence $^{127}\text{Lys-Lys-Lys-Arg-Lys}^{131}$, thereby potentially allowing the conversion of each of these residues to any of three different amino acid residues. Thus the oligonucleotide mixture contained 4^5 components, one of which was an exact copy of the wild-type SV40 large-T coding sequence in this region and 15 of which contained a single base change. The oligonucleotide mixture was incorporated into plasmid DNA bearing a cloned wild-type large-T gene by direct ligation into a gapped heteroduplex, formed between wild-type plasmid DNA and two restriction fragments of a linker insertion/deletion mutant derivative of the plasmid (Fig. 1).

After transfection, colony hybridization¹⁷ using a 19-nucleotide long probe of sequence corresponding to the wild-type gene in this region (4,423–4,441: ACCTTTCTCTTCTTTT) was used to detect plasmids bearing mutations within this sequence. Washing filters at increasing temperatures¹⁸ following hybridization at room temperature clearly distinguished between wild-type plasmids in which hybrids were stable up to about 55 °C (in 6×SSC) and others which showed no detectable hybridization beyond about 45 °C. Although this latter class could itself be resolved into two distinct categories because of differences in the stability of hybridization to the oligonucleotide probe, plasmid DNA was isolated from all poorly hybridizing colonies and examined by restriction enzyme mapping. All plasmids for which hybrids were not stable above ~37 °C, contained large

Table 1 Biological properties of mutant large-T proteins produced by mixed oligonucleotide mutagenesis

Wild-type plasmid						Transformation Focus formation on Rat-1 cells				Replication		Location of large-T in transformed Rat-1 cells
	127	128	129	130	131	10 µg DNA		1 µg DNA		Plasmid DNA	Virus	
	Lys	Lys	Lys	Arg	Lys	Expt	% wt	Expt	% wt			
X46	Thr					(1)	59	(A)	67	—	—	Nuclear
						(2)	73	(A)	80			
d2	Ile					(3)	28	(A)	135	—	—	Nuclear
						(4)	112	(C)	164	—	—	
d10		Thr				(6)	107	(A)	140	—	—	Cytoplasmic
						(7)	101					
X12		Thr				(2)	16	(A)	83	—	—	Cytoplasmic
						(4)	48					
d13			Thr			(3)	14	(A)	40	+	D*	Mixed
						(5)	23	(B)	61			
d1			Met		Thr	(3)	190	(A)	105	—	—	Mixed
						(7)	96	(E)	378			
X18				Ile		(1)	54	(A)	54	—	—	Mixed
X7				Thr		(2)	33	(A)	31	D†	D‡	Mixed
X8					Arg	(1)	56	(A)	37	+	+	Nuclear
d27					Thr	(3)	24	(D)	74	+	+	Mixed
						(4)	40	(C)	103			

Transformation assays: Plasmid DNA (10 µg or 1 µg + 9 µg salmon sperm DNA) was transfected into semi-confluent Rat-1 cells by the calcium phosphate method²⁰. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Dense foci were visible after about 14 days post-transfection and were counted 3–9 days after their first appearance. The numbers above represent the mean of duplicate assays expressed as a percentage of the number of foci induced by the wild-type plasmid, pPVU-0, in the same experiment. The numbers of foci induced by pPVU-0 in these experiments were as follows: Experiment (1) 1,220; (2) 1,150; (3) 446; (4) 500; (5) 464; (6) 694; (7) 634. (A) 170; (B) 94; (C) 50; (D) 76; (E) 62. The deletion mutants RL6 and S5-S32 (ref. 9) which encode a wild-type small-t but severely truncated large-T proteins of 175 and 142 amino acids, respectively, failed to induce any foci in these assays.

Replication assays: Plasmid DNA and viral replication assays were performed as described elsewhere⁹. (—) Indicates that no DNA was detectable in an experiment that would detect 0.1% of wild-type DNA accumulation or that no plaques were detectable within 4 weeks (wild-type plaques are normally visible by 10 days); (+) indicates behaviour indistinguishable from wild-type and (D) (defective) behaviour intermediate between these extremes.

Subcellular localization: Cells derived from transformed foci of Rat-1 cells were grown on glass coverslips, fixed in 4% formaldehyde in phosphate-buffered saline (20 min at room temperature), permeabilized with 1% Nonidet P40 (5 min at room temperature) and incubated with hamster anti-tumour serum followed by fluorescein isothiocyanate-labelled rabbit anti-hamster antibody (Nordic). d10 and X12 transformed cells were also examined using monoclonal antibody PAb423 (ref. 21), followed by rhodamine isothiocyanate-conjugated rabbit anti-mouse antibody. Controls using normal Rat-1 cells and normal hamster serum were included. Normal Rat-1 cells were also generally still present in the population of cells derived from dense foci and thus acted as internal controls for non-specific staining. Immunofluorescence was scored as nuclear, cytoplasmic or mixed. Mixed indicates that three different patterns of staining could be seen in individual cells within a population of transformed cells. Some cells were stained only in the nucleus, others only in the cytoplasm and the rest in both compartments. The most extreme example of this phenotype is d1. Similar localization patterns were seen by staining with the large-T specific monoclonal antibody PAb423 following manual microinjection of each of the mutant plasmids into Vero cells.

* Plaques visible 3 days later than wild-type. † Less than 10% of wild-type DNA accumulation. ‡ Plaques visible 6 days later than wild-type.

deletions (about 2 kb) that included the target for mutagenesis. In contrast, all plasmids for which hybrids were stable to between 40 °C and 45 °C were of wild-type size, indicating the presence of point mutations within the target area. This was confirmed by determining the DNA sequence in this region of all plasmids which formed hybrids of intermediate stability, using the oligonucleotide primed dideoxy procedure applied to double-stranded DNA¹⁹.

Nine of the eleven plasmids sequenced in this way were found to have single point mutations of the wild-type sequence at positions corresponding to the degeneracies in the oligonucleotide mixture (Fig. 2). One mutant plasmid (d1) contained two such alterations and another contained an A to C transversion at a position that should have been invariant (and wild-type) in the oligonucleotide mixture. Two independent isolates of one mutant plasmid (X12; d10) were recovered. In a subsequent experiment using the same oligonucleotide mixture, three further single point mutants were isolated (W. H. Colledge, personal communication). Thus, 12 of the 15 possible point mutants have been obtained. Mutants were recovered corresponding to each of the degenerate positions in the oligonucleotide mixture and examples of each kind of mutagenic change (A → C, G, T) were found. The overall efficiency of production of point mutants was enhanced from 0.7% to 3% by the inclusion of an S₁ nuclease digestion step immediately before transfection, designed to linearize those gapped heteroduplexes to which no oligonucleotide had hybridized. The ease with which large numbers of plasmids can be reliably screened for the presence of point mutations by hybridization to an oligonucleotide of wild-type sequence compensates for the relatively low rate at which mutant plasmids are produced. The genotype of the mutants recovered demonstrates that the mutagenic procedure selects for the incorporation of synthetic oligonucleotides containing single deviations from the wild-type sequence, from a large

excess of oligonucleotides with multiple alterations. The principle of mixed oligonucleotide mutagenesis should be applicable to all methods of conventional oligonucleotide-directed mutagenesis currently in use¹⁶.

Transforming activity

The transforming potential of mutant plasmids was assayed by measuring the induction of dense foci on a monolayer of Rat-1 cells following calcium phosphate transfection²⁰. The formation of dense foci was large-T dependent, as shown by the failure of deletion mutants that encode a wild-type small-t but severely truncated large-T proteins, to produce dense foci (Table 1). However, all point mutants examined showed a transforming activity similar to wild-type. Although slight differences in specific transforming activity were observed, the latent period before foci became visible was never significantly different from wild-type (13–14 days).

Subcellular localization

Individual foci of transformed Rat-1 cells were picked, the cell population expanded and the subcellular location of large-T assessed by indirect immunofluorescence of permeabilized fixed cells using serum from hamsters bearing SV40-induced tumours as the first antibody.

Rat-1 cells transformed by wild-type large-T, encoded by the plasmid pPVU-0, exhibited a typical nuclear staining with no detectable nucleolar or cytoplasmic staining (Fig. 3a). A strikingly different staining pattern was seen for cells transformed by the point mutants, d10 and X12, both of which encode a threonine residue in place of Lys 128 (Fig. 3c). The nuclei appear dark against a bright cytoplasmic background. An identical pattern was seen using a monoclonal antibody (PAb423; ref. 21) directed to the carboxy-terminal region of large-T as first

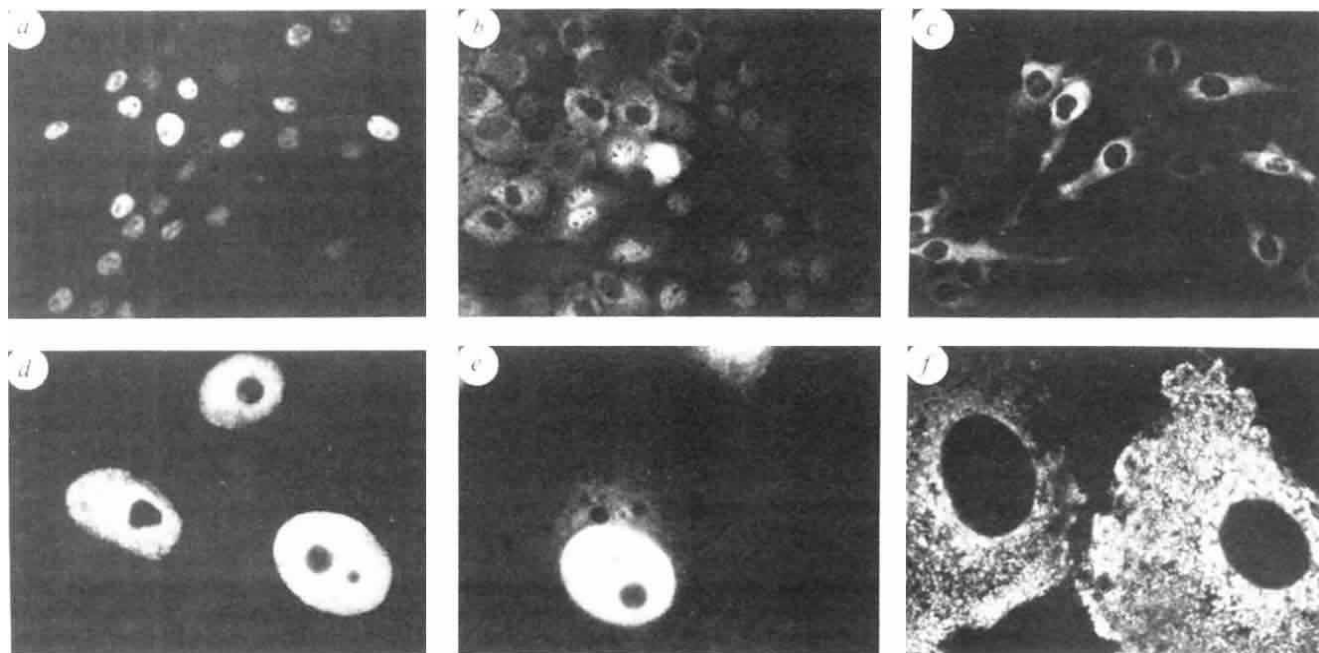


Fig. 3 Nuclear accumulation of large-T in transformed Rat-1 cells and in Vero cells is abolished by alteration of Lys 128 to Thr and impaired by mutation of the surrounding basic amino acid residues. Individual dense foci of Rat-1 cells transformed by wild-type pPVU-0 (panel *a*), the double point mutant d1 (Lys 129→Met, Lys 131→Thr) (panel *b*) and the point mutant d10 (Lys 128→Thr) (panel *c*) were picked and expanded. Cells transformed by d1 were then cloned successively by passage through soft agar and by dilution. Cell lines were fixed and stained by indirect immunofluorescence as described in the legend for Table 1, using hamster anti-tumour serum as the first antibody. The location of large-T in Vero cells was examined by the same indirect immunofluorescence procedure using the monoclonal PAb423 as first antibody, 18 hr after manual microinjection²² of about 200 copies of plasmid DNA into the nucleus of confluent cells growing on glass coverslips. The plasmids injected were wild-type pPVU-0 (panel *d*), d1 (panel *e*) and d10 (panel *f*). The fields shown in *d*–*f* are of confluent monolayers and include some cells that have not been microinjected and show no immunofluorescent staining.

antibody. This antibody, unlike anti-tumour serum, does not recognize SV40 small-t antigen which should be expressed in wild-type form by all mutant plasmids used in this study. The same cytoplasmic staining pattern was seen in Rat-1 cells transformed by d10 that were isolated as colonies growing in soft agar.

The exclusion of large-T from the nuclei of transformed Rat-1 cells consequent to mutation of Lys 128 is not observed for similar mutation of individual adjacent amino acid residues that form part of the five residue basic tract (¹²⁷Lys-Lys-Lys-Arg-Lys¹³¹). Cells transformed by each of these mutants exhibit predominantly nuclear staining. In some cases (d2, X8, X46), the subcellular distribution of large-T appears indistinguishable from that of the wild-type protein. In other cases (X7, X18, d13, d27), a slight defect in nuclear accumulation of large-T is manifest by the presence of some cytoplasmic, in addition to nuclear, staining in the majority of cells derived from an individual focus. Furthermore, a minority of cells from the same focus show predominantly cytoplasmic staining.

The heterogeneous staining pattern (Fig. 3*b*) is even more obvious for the double mutant, d1 (Lys 129→Met, Lys 131→Thr). To date, the subcellular distribution of d1 large-T has been examined in 16 foci, derived from two independent transformation assays. In only one of these was a homogeneous staining pattern observed, with the mutant large-T apparently confined to the cytoplasm of the transformed cells. In all other cases, nuclear staining exceeded cytoplasmic staining in the majority of cells (60–95%); some cells clearly exhibited both nuclear and cytoplasmic staining, whereas in others large-T was apparently either wholly cytoplasmic or wholly nuclear. Cells derived from three of these foci were cloned further either by limiting dilution or by passage through soft agar. In each case, the heterogeneous staining pattern was preserved, indicating that clonally derived sibling cells express a large-T antigen that can, at any one time, be present predominantly in either cytoplasmic or nuclear compartments.

The variable distribution of the large-T protein encoded by the mutants d1, d13, d27, X7 and X18 shows that mutations close to Lys 128 can modulate but do not prevent the accumula-

tion of large-T in the nucleus. Although other interpretations are possible, the heterogeneous localization of large-T in cells transformed by these mutants may reflect a deficiency in the rate of nuclear accumulation less extreme than that observed for d10, which allows the visualization of intermediate stages in the nuclear accumulation of large-T, for example following the breakdown of the nuclear envelope at mitosis.

Transient expression

The wild-type plasmid, pPVU-0, and mutant derivatives were manually microinjected into the nucleus of semi-confluent Vero cells²² and the location of the encoded large-T products assessed by indirect immunofluorescence 18 h post-injection. The staining patterns observed were virtually identical to those seen for transformed cells but were of much greater intensity, presumably due to the large number of gene copies available for transcription. Thus, large-T coded by wild-type pPVU-0 was exclusively nuclear (Fig. 3*d*) and that encoded by d10 was seen only in the cytoplasm (Fig. 3*f*). Both nuclear and cytoplasmic staining was observed for the mutants d1, d13, d27, X7 and X18 (Fig. 3*e*) but the heterogeneity in this staining pattern between individual cells in a population was not as marked as for transformed cell lines.

Replicative activity

As all mutations were introduced into a plasmid, pPVU-0 (ref. 23) that contains the viral origin of DNA replication as well as the entire large-T gene, the ability of the mutant large-T proteins to stimulate the replication of viral origin-bearing episomes in permissive cells could be tested directly by transfection of mutant plasmids into CV-1 cells and quantitation of intracellular plasmid DNA 72 h later²⁴. In addition, the mutant large-T coding sequences from these plasmids were reconstructed into viral DNA and the viability of mutant virus tested by plaque assay²⁵. The two assays gave similar results (Table 1), indicating that several of the mutants were unable to support viral DNA replication. Many other mutants⁹ with lesions either side of those

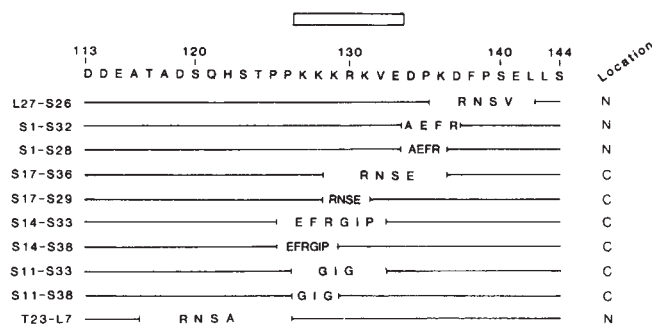


Fig. 4 Amino acid sequence of large-T proteins with deletions in the vicinity of Lys 128. Mutants were constructed from wild-type pPVU-0 containing one or two *EcoRI* linkers CGGAATTCG at the site of a small deletion⁹. The consequent inferred alterations to the wild-type amino acid sequence are shown. Subcellular localization of mutant large-T antigens in transformed cells was determined as in Table 1 and Fig. 3. (+) (–) indicate the presence or absence, respectively, of nuclear immunofluorescence. Some of the large-T coded by T23-L7 was also detected in the cytoplasm. The box above the amino acid sequence of large-T indicates those residues which cannot be deleted without preventing nuclear localization of the mutants described here. Abbreviations used are: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine.

described here also appear to be unable to stimulate viral DNA replication, indicating that this is a common phenotype of mutants from this region.

The viability of mutants altered at Lys 129 and Lys 131, respectively, implies that these (heterogeneously localized) mutant large-T antigens must be able to interact effectively with DNA sequences around the viral origin of replication. Direct measurement of DNA-binding activity *in vitro* by the McKay assay²⁶ suggests that the non-viable cytoplasmic (d10) and partially cytoplasmic (d1) mutants can also recognize SV40 origin DNA (E. Paucha, D.K. and A.E.S., manuscript in preparation), suggesting that the aberrant localization of these proteins is not related to their ability to interact with SV40 DNA.

Sequences around Lys 128

The data above show that alteration of Lys 128 and, to a lesser extent, the surrounding basic amino acid residues either modulates or abolishes the nuclear accumulation of large-T. The properties of a large number of single, double and triple point mutants with lesions either side of this region between amino acids 106 and 158, by contrast, do not exhibit any impairment in the nuclear accumulation of large-T⁹. The importance of Lys 128 to the localization of large-T is highlighted further by the recent observation of R. G. Lanford and J. S. Butel (personal communication) that the only lesion in the large-T coding sequence of the PARA-cT variant²⁷, isolated several years ago on the basis of its cytoplasmic phenotype, is the conversion of the codon for Lys 128 to that for Asn.

It is likely, therefore, that Lys 128 forms an essential part of some structural feature of the wild-type large-T protein that allows it either to be transported to or retained within the nucleus. The other possibility that the variant large-T proteins coded by d10 and PARA-cT have acquired an affinity for cytoplasmic components seems improbable, in that a novel specificity of binding is unlikely to be endowed by two different amino acid changes.

The context in which Lys 128 serves to localize large-T to the nucleus has been probed further by examining the properties of a number of insertion/deletion mutants with lesions in the immediate vicinity of Lys 128 (Fig. 4). These were made by standard procedures as part of a more comprehensive mutagenic analysis of large-T⁹. As all of the mutants shown in Fig. 4 were able to transform Rat-1 cells (Table 2), the subcellular distribution of the mutant large-T antigens could be examined in cells

Table 2 Transforming activity and subcellular localization of large-T proteins with deletions in the vicinity of Lys 128

	Net change in no. of amino acid residues	Nuclear large-T	Transformation of Rat-1 cells	
			No. of foci % wt 10 µg DNA	No. of foci % wt 1 µg DNA
L27-S26	–3	+	81	44
S1-S32	0	+	3, 20, 35*	30
S1-S28	+1	+	30	40
S17-S36	–4	–	66, 50	36
S17-S29	+1	–	38, 53	17
S14-S33	+1	–		
S14-S38	+2	–		
S11-S33	–3	–	72	75
S11-S38	0	–	110	35
T23-L7	–6	+	21	75

The transforming activity and subcellular locations of the mutant large-T proteins in transformed cells is given. These parameters were assayed as described in Table 1.

* These foci appeared 7, 3 and 6 days later than wild-type, respectively.

derived from transformed foci. In all cases where any part of the basic tract of amino acid residues (127–131) is interrupted, even if this does not include Lys 128, large-T is seen only in the cytoplasm of transformed cells (Fig. 4). However, the normal nuclear accumulation of large-T was not detectably impaired by the alteration of as many as four consecutive amino acid residues distal to Glu 133 and was only marginally affected by changing as many as ten residues proximal to Lys 127. Thus, if there exists a region of primary amino acid sequence around Lys 128 that directs large-T to the nucleus, it probably does not extend beyond Lys 127 or Glu 133. The tolerance of the nuclear large-T mutants L27-S26 and T23-L7 to variation in the length of polypeptide chain separating this sequence from the rest of the molecule perhaps also suggests that the function of Lys 128 is not dependent on a precisely structured three-dimensional configuration within the protein. The cytoplasmic location of several different insertion/deletion mutants supports the argument presented earlier that this phenotype does not result from a novel retention of large-T in the cytoplasm but rather from a defect in transport to or retention within the nucleus.

All of the cytoplasmic insertion/deletion mutants, like d10, transform Rat-1 cells according to dense focus formation in high serum with an efficiency and latent period similar to wild-type (Table 2).

Transformation by non-nuclear T

SV40 large-T is an unusual transforming protein in that it can transform primary cells as well as established cell lines²⁸ and gives rise to a gradation of transformed phenotypes²⁹. In this study only the ability of large-T to transform an established cell line, Rat-1, in high serum was measured by the criterion of dense focus formation. None of the cytoplasmic large-T deletion or point mutants showed a significant deficiency in transforming activity relative to wild-type in this assay. Preliminary experiments indicate that the point mutant, d10, also transforms Rat-1 cells, by the criterion of colony formation in soft agar at high serum concentration with an efficiency similar to wild-type. However, any implication that large-T need not be nuclear to transform, must be tempered by two considerations. First, although large-T is seen by immunofluorescence only in the cytoplasm of cells transformed by d10 or by any of several deletion mutants, some large-T may nevertheless be present in the nucleus but remain undetected, either because of its very low abundance or because it is in a form that is not accessible to the antibodies used for its detection. Second, it is possible that differences in the transforming activity of nuclear and non-nuclear large-T species may be revealed by more stringent assays. By using such assays, R. G. Lanford and J. S. Butel

concluded that the cytoplasmic large-T encoded by the PARA-cT mutant induces a partially transformed phenotype in hamster cells, although it can induce colony formation in semi-solid medium and tumour formation *in vivo*³⁰. In view of the notion that the transformation of primary cells involves the combined action of a nuclear immortalizing gene product (such as polyoma virus large-T, adenovirus Ela protein, the *myc* gene product) and a membrane-associated protein³¹ (such as the *ras* gene product, polyoma virus middle-T), it might be predicted that a non-nuclear large-T would be unable to immortalize or transform primary cells, whereas the plasma-membrane associated form of large-T⁵ may suffice to transform established cell lines. These predictions are being tested using the cytoplasmic mutants described here.

Mechanisms of nuclear localization

The nuclear envelope differs from other cellular membranes in that it allows the free passage of virtually all proteins below a certain size (corresponding to approximately 18,000 M_r) in addition to some larger proteins³²⁻³⁴. Thus, for any given protein it is not clear whether nuclear accumulation results from a specific mechanism that either facilitates or drives transport across the envelope or whether the protein is free to diffuse, for instance through nuclear pores and is then retained in the nucleus by virtue of an affinity for elements that are themselves confined to the nucleus. This distinction has not been made for SV40 large-T, though the large size of the monomer may favour a specific transport mechanism.

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The accumulation of nucleoplasmin in the nucleus of *Xenopus laevis* oocytes³⁵ is the only case for which there is good evidence for the involvement of a nuclear transport system. Proteolytic digestion of the native pentameric protein has shown that a 16,000 M_r fragment derived from a single 33,000 M_r monomeric unit is sufficient to allow the nuclear accumulation of partial proteolytic cleavage products as large as 101,000 M_r , and is therefore presumed also to specify the nuclear location of the native protein.

Whatever the mechanism of nuclear accumulation, the evidence presented here heralds the possibility that a very restricted region of the SV40 large-T amino acid sequence around Lys 128 may be sufficient to ensure the nuclear location of this protein. Further experiments are in progress to determine more precisely to what extent this putative signal is independent of the rest of the large-T molecule and whether the signal can act in the context of other proteins that are normally cytoplasmic so as to direct them to the nucleus. The availability of the d10 mutant will be invaluable as a control for such studies. When this manuscript was in preparation, M. N. Hall *et al.* reported that restricted sequences derived from the yeast Mat $\alpha 2$ gene product can direct another protein to the nucleus in yeast³⁶.

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LETTERS TO NATURE

The boundary of the Solar System

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The shape of the boundary of the Solar System, defined as the surface within which the gravitational attraction of the Sun, rather than that of the rest of the Galaxy, controls the orbital motion of bodies such as planets and comets, has been determined. Outside of this surface, the dominant factors are the radial tides due to the galactic centre and, especially, the vertical tides caused by the galactic disk. Orbits which are direct with respect to the galactic plane, have a boundary which differs from that for retrograde orbits, both being 10-20% oblate and both larger than the, usually assumed, present Oort cloud. The surface may have been the boundary of the early cloud of comets which was later reduced by the passages of stars and molecular clouds.

Losses of bodies from the Solar System are caused by: sporadic perturbations by passing stars or giant molecular clouds, which occur at a rate of several per 10^6 or 10^9 yr respectively, perturbations by giant planets and the steady gravitational perturbation by the galaxy. The first two kinds of perturbations have been extensively studied¹⁻⁴. We now describe the results of a three-dimensional stability study (inertial coordinates) of a system consisting of a comet and the Sun rotating⁵ around the galactic centre [mass $1.3 \times 10^{11} M_\odot$, distance 8.2 kpc (ref. 6)] and periodically traversing the nearly harmonic field of the galactic mid-plane (GMP)⁷. A two-dimensional model of a greatly simplified version of this problem has been studied previously⁸ and a corresponding three-dimensional Hill surface is also known⁸. To get an indication of the extent and structure of the boundary of stability⁹ paths of comets were integrated numerically for various initial parameters using Cowell's method and fourth-order Runge-Kutta integration. Near the boundary of stability the orbit of a comet is non-keplerian, its semimajor axis a , average inclination i , eccentricity e and the direction of perihelion $\tilde{\omega}$ are highly variable. Thus, for an initial i , the values of e and a were so chosen as to obtain an orbit which was bound for the age of the Solar System. The value of $\tilde{\omega}$ had essentially no effect on the results.

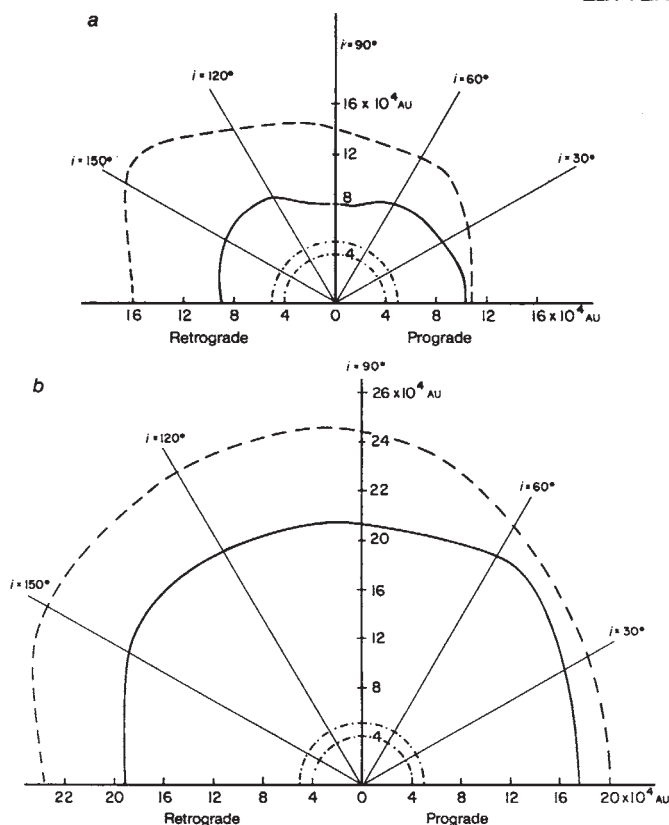


Fig. 1 *a*, Maximum stable semi-major axes of orbits perturbed by the galactic centre alone (dashed line) and by the galactic centre and the mid-plane (solid line). The usually assumed size of the Oort cloud is also indicated (dash-dot line). *b*, Maximum aphelia of orbits perturbed.

The effect of the galactic centre was investigated first and then the influence of the GMP was included. Without such an influence, a cometary orbit is, in general, more stable for high eccentricities and inclinations near 90° as it then senses less of the tidal field of the galactic centre¹⁰ and thus the stability boundary has a proloid shape as shown by dashed lines in Fig. 1. When the effect of the gravitational compression⁷ caused by the oscillations of the Sun through the GMP is added, the results indicate a strong destabilization of all orbits especially for i higher than 90° . As shown in Fig. 1, the boundaries of stability then become oblate by 10–20%.

For large a , the periods of orbits are comparable to a substantial fraction of 1.9×10^8 yr, the period of the Sun around the galactic centre or to the shorter period of motion of the Sun across the GMP, so that the directions of the galactic tidal forces change significantly and a resonance can occur between the period of the comet and the other two. The three-dimensional shape of the limits of the stable Solar System, taking both the GC and GMP into account, is obtained by rotating the curves shown in Fig. 1 around the z -axis perpendicular to the plane of the galaxy. This leads to two rather similar co-axial surfaces: the inner one, enclosing the prograde orbits and the outer one, limiting the extent of the retrograde orbits. A rough estimate of the volume of these surfaces suggests that if the density of comets per unit volume were uniform, there would be ~20% more retrograde than prograde comets. The assumption of a uniform density is, however, questionable. Both surfaces have dimensions that are appreciably larger than the commonly assumed radii of the Oort cloud whether based on observation or on other theoretical estimates^{1,2,10–13}.

Among the stars which penetrate and perturb the Oort cloud those in the mass range of 0.15 – $0.85 M_\odot$, have higher velocities than those with masses in the 0.85 – $1.15 M_\odot$ range. Taking the mass¹⁴ and velocity distribution into account indicates that the velocity impulse produced by one of the smaller stars is, on average, one-third of that caused by a more massive star. The

smaller stars are, however, sufficiently more numerous so that the net perturbations are comparable. The frequency of encounters with the region of stable pro- and retrograde orbits is about 10 per 10^6 yr while for the present Oort cloud it is, depending on the assumed radius, 2 – 6 per 10^6 yr.

Comets spend most of the time near their aphelia where they are weakly bound and thus the outer regions of both clouds will be severely depleted in spite of continuous redistribution of comets by weak perturbations. It is possible that the early Oort cloud, whatever its origin was comparable in size to the volume of stability here described or, if it were initially smaller, it would have been spread by early perturbations. Later perturbations especially by molecular clouds^{3,15,16} decreased the cloud to its present size with a roughly comparable number of prograde and retrograde comets. Such a sequence of events may have an important role in explaining the primordial bombardment of the Solar System. An estimate of the total number of comets in this early cloud and its subsequent changes is beyond the scope of this letter.

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Emission of large hydrocarbons from frozen CH_4 by keV proton irradiation

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Ever since the first description of the synthesis of molecules by impinging protons on ices¹, the possibility of formation of various molecules on cold surfaces by particle radiation has been widely discussed^{2–5}. Because fluxes of light ions with energies of ~ 1 keV AMU^{-1} exist in a wide variety of astrophysical sites⁶ there has been a dispute over whether molecules in space are formed by such a process or by gaseous reactions⁷. We have recently studied sputtering of species by bombarding simple frozen gases such as water, carbon monoxide and ammonia with light ions². With these substances, only rather simple molecules were found. However, when bombarding methane, we detected much larger molecules in the sputtered beam—hydrocarbons containing up to at least 13 atoms. We propose, therefore, that bombardment of frozen methane by keV protons may produce large molecules in the Solar system.

Frozen methane at 15 K was bombarded with 6-keV H_2^+ ions and the sputtered flux was analysed by mass spectrometry. The frozen layer had a thickness of ~ 10 μm (much larger than the range of the projectiles). The ion-beam flux was 50 – 100 $\mu\text{A cm}^{-2}$.

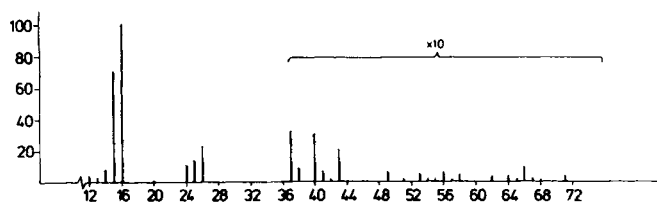


Fig. 1 Mass spectrum of the neutral species sputtered from frozen methane at 15 K by 6-keV H_2^+ ions.

H_2^+ ions were chosen instead of H^+ ions because of achievable ion beam intensity, and on entering the target surface, the H_2^+ ions dissociate at the first collision.

The kinetic energy of the sputtered species was determined by a time-of-flight method². Sputtering of various hydrocarbons was inferred from the spectrum shown in Fig. 1. This gives the signals found in phase with the ion beam modulation, so that continuous background contributions, including those from evaporation, are subtracted.

The signals of sputtered species drastically decrease as the size of molecules increases, and are too low to detect when molecules contain more than five carbon atoms. On the other hand, the total sputtering yield in these experiments is about 1–10, that is, for every incoming ion a few neutral molecules are sputtered. The low fluxes of the larger molecules prohibited time-of-flight measurements, but this was possible for C_2H_2 . We found a median energy of 0.063 eV, although 10% of the sputtered C_2H_2 had an energy of >0.28 eV. These energies are far in excess of what can be expected for a volatile substance at 15 K. In addition, the energy distribution is not Maxwellian and agrees with that of a collision cascade mechanism².

After bombardment, a residue was found which was later warmed up and analysed by pyrolysis mass spectrometry⁸. The resulting spectrum, (Fig. 2) shows masses of up to about 170 indicating that the residue contains molecules with at least 12 carbon atoms. The technique used was the so-called 'open-system flash-pyrolysis mass spectrometry' in which the products from the pyrolysis immediately escape and cannot react with the substrate. Thus synthesis cannot take place but fragmentation clearly does. Note that during the initial warming up of the bombarded layer to room temperature, reactions may have occurred and, therefore, the results from the pyrolysis mass spectrometry only hint that larger molecules may be present in the bombarded sample.

In both the sputtered flux and the residue the largest molecules found were present in concentrations approaching the limit of

the sensitivity of our measurements, and thus even larger molecules may have been formed and sputtered by a collision-cascade mechanism. Obviously some polymerization takes place in the process, producing very large molecules. Polymerization of CH_4 may be caused by electrical discharges, UV light or MeV protons⁹, and we conclude from the present measurements that large molecules are also formed by keV ions and infer that a considerable fraction of these is sputtered.

Note that such a process may occur on the most outer planet Pluto and its moon Charon which are covered with methane ice^{10,11}. The escape velocity v is given by $v = (2GM/R)^{1/2}$ in which M , R and G are the mass and radius of the planet and G is the gravitation constant, $6.6732 \times 10^{-11} \text{ N m}^2 \text{ kg}^{-2}$. For Pluto $M = 1.13 \times 10^{22} \text{ kg}$ and $R = 1,800 \text{ km}$ and the escape velocity becomes 917 m s^{-1} (<10% of that of the Earth). The escape energy is then $0.0043 \text{ eV AMU}^{-1}$. For Charon $M = 1.0 \times 10^{21} \text{ kg}$ and $R = 800 \text{ km}$. This gives an escape energy of $<0.001 \text{ eV AMU}^{-1}$. Therefore, large molecules formed and sputtered from these small planets may turn up in the Solar System. From the above measurements we can infer that the total flux of sputtered molecules is of the same order of magnitude as the incident flux of the solar wind.

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Diffuse reflectance pulse radiolysis of opaque samples

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Flash photolysis and pulse radiolysis techniques¹ have contributed substantially to our understanding of the harmful and beneficial effects of radiation. The development of extremely short pulsed excitation sources means that time resolution is no longer a serious limitation to the application of these methods^{2,3}. However, these methods have previously been restricted to transparent samples because changes in transmission of analysing light were measured following pulsed excitation. Recently^{4–6}, we have established that photochemical processes within opaque and highly scattering systems can be studied by observing changes in diffusely-reflected analysing light following laser excitation. Many of the most important radiation-induced processes involve non-transparent, heterogeneous systems, for example, most biological systems. We report here the first successful 'diffuse-reflectance pulse radiolysis' experiments in which we demonstrate that time-dependent absorption spectra of short-lived species can now be measured for opaque samples following pulse radiolysis.

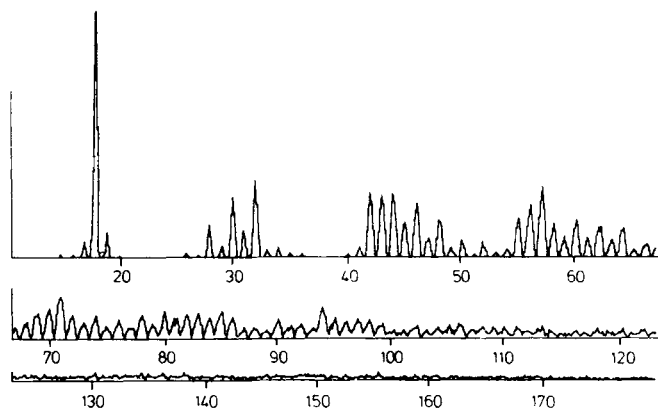


Fig. 2 Pyrolysis mass spectrum of the residue obtained from frozen CD_4 by bombardment with 6-keV H_2^+ ions after warming up to room temperature.

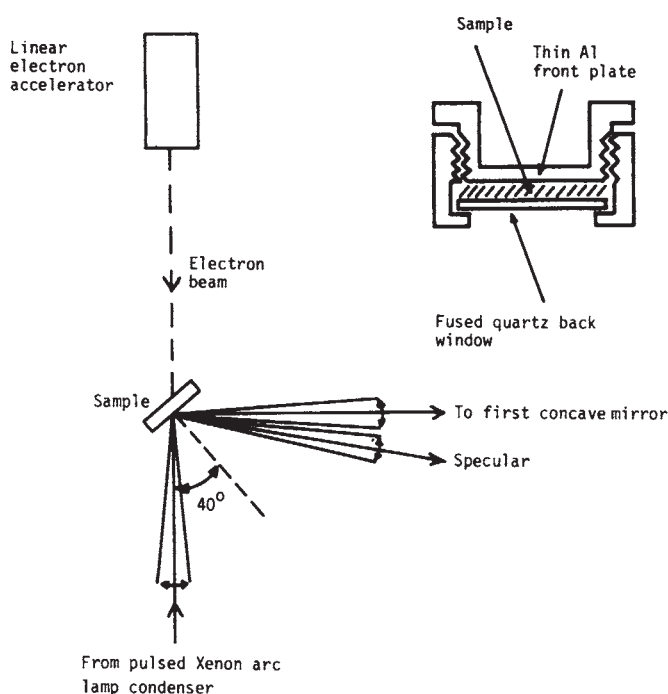


Fig. 1 Schematic diagram of the equipment used for studying diffuse reflectance pulse radiolysis of opaque samples. (Inset shows sample holder in more detail.) Diffusely reflected monitoring light diverging from the sample into a cone of solid angle 0.07 sr is imaged on to the entrance slit of a monochromator (outside the accelerator area at unit magnification) by means of two concave mirrors of 23-cm diameter and 77-cm focal length spaced about 10 m apart.

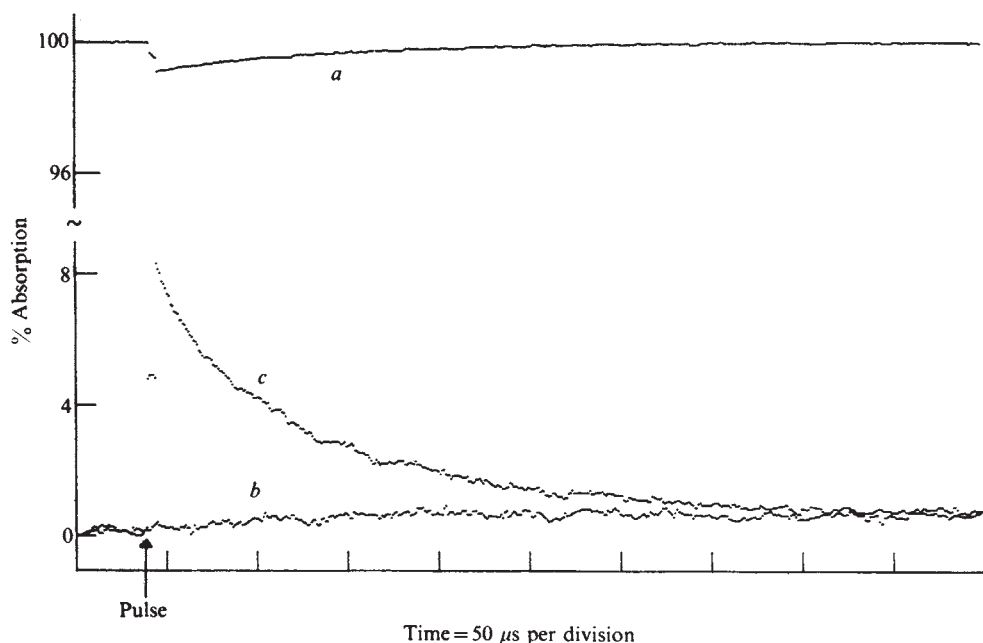
The pulse radiolysis apparatus has been described previously⁷. Typically 200 ns pulses of 10-MeV electrons were used which delivered doses of up to 100 Gy to the sample through a thin aluminium front plate. Analysing light was obtained from a pulsed xenon arc⁸ which was triggered $\sim 750 \mu\text{s}$ before the excitation pulse fired. This gave a steady reproducible intensity of analysing light which was reflected off the sample through the optically-transparent 'back' face of the irradiation sample

holder (see Fig. 1). Collection of specular reflected light with angle of incidence equal to angle of reflection was avoided as far as possible as it contains little transient information. 10-MeV electrons in the excitation pulse are not strongly attenuated on passing through the opaque samples which typically had path lengths of ~ 1 mm. Thus, 'back' face analysis, which did not require major modification of the pulse radiolysis apparatus designed for normal transmission detection, proved successful (see Fig. 1). This contrasts with the situation when excitation is by pulses of light which are usually substantially attenuated, resulting in a much smaller penetrating depth. Thus, with light excitation, analysis of diffusely-reflected analysing light at the front irradiated surface is essential. The diffusely-reflected light before pulse radiolysis gives a perfect blank in these experiments. A Tektronix 7912 transient digitizer was used to capture the transient information which was then transferred to a minicomputer for analysis. Figure 2 shows a typical set of traces for pulsed radiolysis of microcrystals of benzil analysed at 510 nm. Trace *c* can be corrected for any observed emission, and, by making measurements at different wavelengths, time-dependent absorption spectra of short-lived species produced by pulsed ionizing radiation can be obtained.

The first experiments were performed with opaque samples of organic microcrystals which have already been shown to give transient intermediates in diffuse reflectance laser flash photolysis experiments. Diffuse reflectance pulse radiolysis of benzophenone and benzil give transient absorption spectra which have similar spectra and similar half lives to those obtained by laser flash photolysis^{5,6}. Figure 3 shows a comparison of the transient absorption spectra for benzophenone observed from pulse radiolysis and laser photolysis. Phosphorescence decay following pulse radiolysis was also measured and differences were observed between the decay of the transient absorption and the phosphorescence in the case of benzil. This may mean that there is more than one transient absorption, possibly there is some contribution from the negative radical ion of this ketone which would be expected to absorb in the same region as the triplet state.

Several other samples were subjected to the technique and most gave weak transient absorption of $< 2\%$. Oxygen was not excluded from any of these samples as the transients observed when microcrystals of benzil and benzophenone are subjected to diffuse reflectance laser flash photolysis are not sensitive to gas phase oxygen. The transient spectrum obtained following pulse radiolysis for naphthalene crystals is shown in Fig. 4a.

Fig. 2 Transient absorption of microcrystalline benzil, excited by 10-MeV electrons (200 ns pulse), monitored at 510 nm, as a function of time. (Sample thickness = 1 mm, dose ≈ 100 Gy, pulse duration ≈ 200 ns.) *a*, The signal observed following excitation in the absence of analysing light resulting from emission at the analysing wavelength. *b*, The diffusely reflected analysing light in the absence of excitation. *c*, The substantial transient absorption of diffusely reflected light following the pulsed electron excitation.



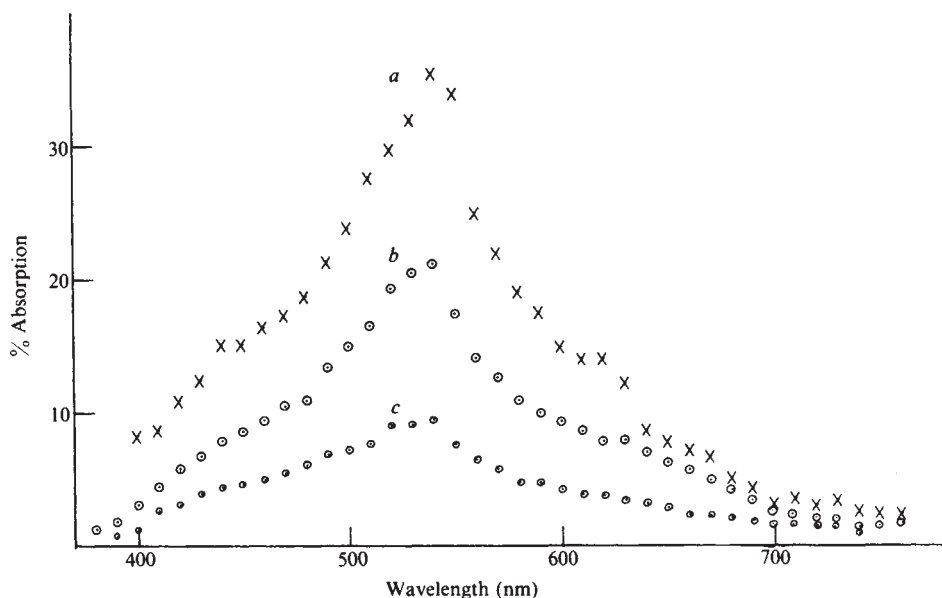
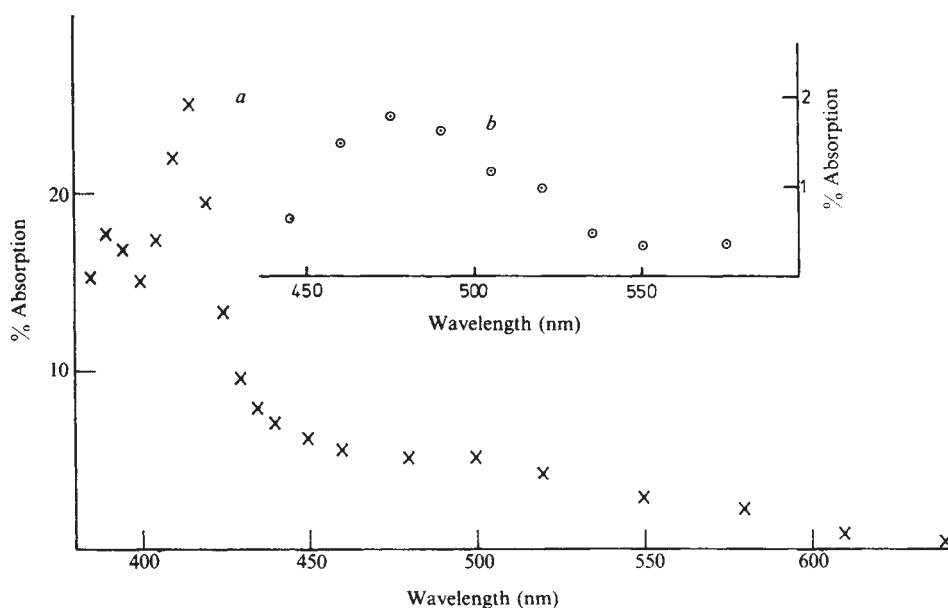


Fig. 3 Excited state absorption spectra of microcrystalline benzophenone. Sample thickness, 1 mm; dose ≈ 100 Gy; pulse duration ≈ 200 ns. *a*, From pulsed laser excitation at 354 nm (ref. 5). *b*, From electron excitation, 3 μ s after the end of excitation. *c*, From electron excitation, 13 μ s after the end of excitation.

Fig. 4 *a*, Transient absorption spectrum of microcrystalline naphthalene, following pulse radiolysis ($t_{1/2} \approx 20$ μ s; sample thickness = 1 mm; dose ≈ 80 Gy; pulse duration ≈ 200 ns). *b*, Transient absorption spectrum of bovine muscle tissue following pulsed radiolysis ($t_{1/2} \approx 20$ μ s; sample thickness = 5 mm; dose ≈ 100 Gy; pulse duration ≈ 200 ns).



The absorption spectra in the range 380–440 nm shows strong similarities to the triplet-triplet absorption spectrum of naphthalene obtained in dilute solution using transmission flash photolysis⁹. We tentatively assign the transient absorption in this region to the triplet state of naphthalene. Note that quite a wide distribution of optical absorption path lengths is possible in different samples and that this should be considered in any interpretation of spectra or kinetics.

We stress that pulse radiolysis using diffusely reflected light for analysis extends considerably the range of systems that can be studied by this technique. Diffuse reflectance spectroscopy has been used to probe the effects of radiolysis on, for example, polycrystalline selenites¹⁰, but we believe our work is the first to use diffusely-reflected light to monitor the decay of excited states produced by pulse radiolysis. We feel that this development has most potential for studies of biological systems which are non-transparent. To illustrate this, we have subjected a sample of bovine muscle tissue to this technique and observed the unassigned transient shown in Fig. 4*b*. We consider that our

preliminary measurements provide an important step in helping to unravel the mechanism of radiation-induced processes in complicated biological systems and for non-transparent systems in general.

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Measurements of hydrogen peroxide in polar ice samples

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Hydrogen peroxide, a powerful oxidant, is believed to be a key component in the oxidation of SO_2 to H_2SO_4 in clouds¹. The first quantitative H_2O_2 measurements in snow, rain, hoarfrost and fog were reported in 1874 (ref. 2), however, systematic investigations of H_2O_2 concentrations in precipitation and hydrometeors began only a few years ago^{3,4}. We report here measurements of hydrogen peroxide in polar ice samples. To our knowledge, chemically-reactive species have not been previously analysed in ice core samples. Our measurements show that H_2O_2 is a dominant trace compound present in clouds over remote and clean areas.

The cores from Greenland were drilled as part of the US-Danish-Swiss Greenland Ice Sheet Program. The North Central core, drilled in 1977, and the Dye 3 deep core drilled in 1979–81 were mechanically drilled and are in perfect condition. The Greenland sample series corresponded to a precipitation period of ~1 yr. The locations from which the samples were taken are listed in Table 1 together with their glaciological characteristics.

The analytical system is described in detail elsewhere⁵. Hydrogen peroxide is measured through the chemiluminescence radiation emitted during its reaction with bis-trichlorophenylloxalic ester (TCPO)⁶. Perylene is used as a fluorescing agent. Both the perylene and the TCPO are dissolved in purified acetone. The reaction between the H_2O_2 solution and the TCPO reagent (acetone to water ratio 3:1) takes place in a mixing chamber placed in front of a photomultiplier. The emission of the chemiluminescent reaction is linear with H_2O_2 concentration in the range between 0.5 p.p.b. (parts per 10^9) (w/w), detection limit and 30 p.p.m. (parts per 10^6) (w/w).

Because the reaction of hydrogen peroxide with TCPO strongly depends on the pH of the solution, the samples were adjusted to a pH of 8 using a borate buffer.

Ice cubes (2–5 cm³) were cut in a cold room (–20 °C) with a band saw, so that at least a 1 cm layer was removed from each surface. The cubes were cleaned with a Teflon brush and placed in polyethylene containers. The ice cubes were melted in the containers in a warm water bath (~40 °C). As soon as a whole cube was melted, a sample was taken, mixed with the buffer, and injected into the analytical system. To check whether the handling introduced any noticeable background contamination, ice cubes from a laboratory-grown pure single crystal, purified three times by zone melting, were analysed. The H_2O_2 concentration of such samples were below or close to the detection limit of 0.5 p.p.b.

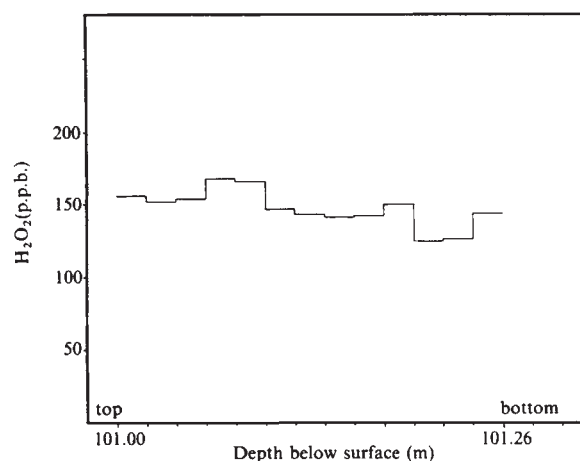


Fig. 1 H_2O_2 concentration along a 26-cm segment from North Central Greenland at a depth of 101 m below the surface. The ice is ~650 yr-old.

Table 1 also shows the measure of mean values of H_2O_2 concentrations with their standard deviation. Figures 1–3 show detailed records from the Dye 3 core and the North Central core.

At Dye 3, melted layers are formed almost every summer⁷. The occurrence of such melted layers may change the H_2O_2 concentration dramatically. In melted layers of the Dye 3 core, concentrations up to 400 p.p.b. H_2O_2 were found (Fig. 2). Figure 2 also shows the distribution of H_2O_2 across a single winter layer of the Dye 3 firm core. A few samples were also analysed from two cores from Antarctica (Byrd and South Pole). (The Byrd core was drilled in 1968–69 and the South Pole core in 1981–82 by the Polar Ice Core Office (PICO).

Because precipitation is stored in the frozen state in ice sheets, chemical trace substances are relatively well preserved and chemical reactions occur extremely slowly. H_2O_2 is produced in the gas phase by the reaction of two HO_2 radicals. The formation of HO_2 radicals in the clean atmosphere starts with the photolysis of O_3 producing $\text{O}^1(\text{D})$, which further reacts with H_2O to form OH radicals⁸. The latter are extremely reactive and several reaction mechanisms, including hydrocarbons and CO as reactants, may lead to the production of HO_2 .

A substantial amount of H_2O_2 , found in cloud droplets, may have been produced in the liquid phase, due to dissolved OH and HO_2 radicals⁹. It is not known whether H_2O_2 is produced or destroyed at the surface of, or inside, snowflakes, either in clouds or on the ground after deposition.

A small amount of H_2O_2 in the ice could have been produced as a consequence of ionization processes caused by cosmic rays. To estimate an upper limit of this production for the South Pole

Table 1 Locations and glaciological characteristics of samples

Station	Geographical location	Mean annual temperature (°C)	Mean accumulation rate (cm water equiv. yr ⁻¹)	Measured samples	Depth below surface (m)	Age (yr BP)	Mean H_2O_2 concentration (µg H_2O_2 per kg ice)
Dye 3	65°11' N 43°50' W	–19.6	50	20	26	30	92 ± 76*
Dye 3	65°11' N 43°50' W	–19.6	50	16	85	115	103 ± 46*
Dye 3	65°11' N 43°50' W	–19.6	50	12	1,724	~9,000	28.9 ± 9.1
Dye 3	65°11' N 43°50' W	–19.6	50	6	1,830	~25,000	<0.5
North central	74°37' N 39°36' W	–31.7	11	13	101	650	149 ± 15
Byrd	79°59' S 120°01' W	–28	16	3	297	1,300	8 ± 0.8
Byrd	79°59' S 120°01' W	–28	16	3	1,652	~22,000	2.8 ± 0.24
South Pole	90° S	–51	7	3	91	650	17.3 ± 1.0
South Pole	90° S	–51	7	3	160	1,600	11.6 ± 0.6
South Pole	90° S	–51	7	3	191	2,000	16.7 ± 1.6

* The standard deviation reflects the seasonal variation and melt layer influence (see Figs 2 and 3).

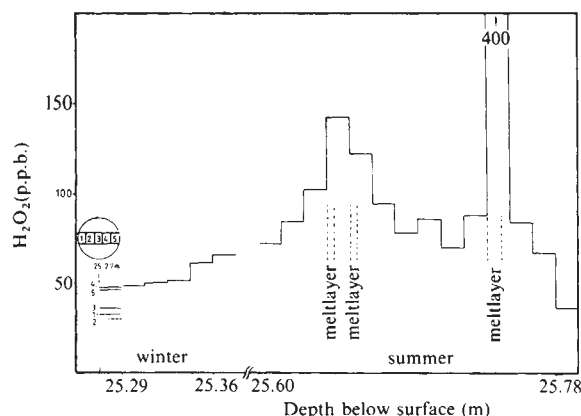


Fig. 2 H_2O_2 concentration in a 30-yr old firn segment from Dye 3 at a depth of 26 m below the surface including melt layers. Summer and winter layers were identified by visual stratigraphy.

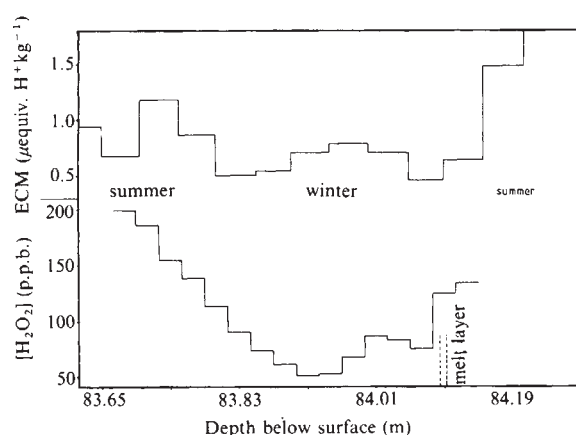


Fig. 3 H_2O_2 concentration and acidity in a 1-yr layer from Dye 3 at a depth of 85 m below the surface, corresponding to an age of 115 yr. Winter and summer layers were identified by the acidity and visual stratigraphy.

core, we assume an ion-pair production of 5×10^3 pairs $\text{g}^{-1} \text{s}^{-1}$ in the ice¹⁰ and H_2O_2 generation of one molecule per ion pair. Furthermore, we assume that the cosmic ray flux is attenuated exponentially with a damping constant of 0.023 m^{-1} in the first 100 m water equivalent of the ice sheet. With this assumption, 5 p.p.b. H_2O_2 could have been built up in 1,500-yr ice from the South Pole. This crude approximation shows that such a mechanism may be of importance at the South Pole. It has little relevance for Greenland however, because here significantly larger H_2O_2 concentrations have been found in the ice cores.

H_2O_2 is one of the dominant trace compounds in the Greenland cores. In comparison, Table 2 shows mean concentrations of other impurities found in Greenland ice¹¹ and South Pole firn¹². The Antarctic samples have a significantly lower H_2O_2 concentration. The annual H_2O_2 deposition rate at Dye 3 would, according to our results, be roughly 30 times higher than at South Pole. This ratio is clearly higher than the one observed for NO_3^- and SO_4^{2-} .

There are three possible causes for the lower H_2O_2 deposition rate at the South Pole:

- (1) Transport of H_2O_2 or precursors from mid-latitude sources. Dye 3 is at a much lower latitude than the South Pole, and is meteorologically connected to the continental regions of America and Europe, whereas the whole Antarctic continent is shielded by the circumpolar vortex from lower latitudes.
- (2) The stratospheric ozone input to the Southern Hemisphere is lower by a factor of about two¹³. The lower stratospheric-tropospheric air mass exchange in the Southern Hemisphere is also evident from measurements^{14,15} of the cosmic ray produced radioisotopes ^7Be and ^{10}Be .

Table 2 Chemical impurities in natural polar ice

Species	Greenland ice sheet* ($\mu\text{mol kg}^{-1}$)	South Pole† ($\mu\text{mol kg}^{-1}$)
Cl^-	0.5	1.25
NO_3^-	1.0	1.44
SO_4^{2-}	0.25	0.75
H^+	1.2	3.37
Na^+	0.4	0.63
$\text{K}^+, \text{Mg}^{2+}, \text{Ca}^{2+}$	0.1	0.2
NH_4^+	0.3	0.16
$\text{H}_2\text{O}_2^\ddagger$	3.2	0.5

* Ref. 11.

† M. Legrand (personal communication), mean values from samples covering the time 1968–78.

‡ This paper.

(3) Snow formation and heterogeneous removal mechanisms may differ at South Pole and Dye 3, especially when the lower temperature at the South Pole is taken into account.

The findings, presented in Figs 2 and 3, may be explained by physical and air chemical processes, respectively. High H_2O_2 levels in melt-layers (Fig. 2) indicate that conditions change, if meltwater is present. However, the change of phase of water from the liquid to the solid state decreases the solubility of dissolved impurities by several orders of magnitude¹⁶. Liquid water in equilibrium with an atmospheric H_2O_2 concentration of 0.5 p.p.b. (v/v), which corresponds to a typical background value⁹, would contain 500 p.p.b. (wt/wt) H_2O_2 . During the freezing process, H_2O_2 will accumulate in the liquid phase adjacent to the freezing front and will gradually be captured in the ice matrix. Concentrations of H_2O_2 in previously melted layers will, therefore, depend on the mode of refreezing. In addition, any chemical reactions that occur while the water is in the liquid state, as well as increased H_2O_2 deposition during the summer, may also alter H_2O_2 concentrations.

The selected core segment from a depth of 83 m below the surface (Fig. 3) contains only a small melt layer. Nevertheless, the H_2O_2 concentration shows a clear variation, which we interpret as a seasonal variation with high H_2O_2 contents in the summer half year and low contents in the winter half year. The summer and winter layers, respectively, were identified by visual stratigraphy and electrical conductivity measurements (ECM), which give the total acidity in ice¹⁷. The ECM results show an almost perfect seasonal variation in the Dye 3 core¹¹. Indeed, model calculations predict a seasonal variation of the H_2O_2 mixing ratio in the atmosphere for high latitudes, with a maximum in summer¹⁸.

Sublimation and recrystallization smooth out such factors as seasonal variations in $\delta^{18}\text{O}$ during the earlier lifetime of the firn from stations with low snow accumulation rates, such as North Central¹⁹. Thus, we did not expect to see a seasonal variation of the H_2O_2 concentration in the North Central core, and this was confirmed by measurements (Fig. 1).

H_2O_2 is a reactive substance and it is not yet known whether chemical conversions occur in ice. Even if they proceed slowly, they could have affected the H_2O_2 concentration during the hundreds of years since snow formation. Samples from the Dye 3 core from 1,724 m below surface, corresponding to an age of 9,000 yr, gave a mean H_2O_2 concentration of 28.9 p.p.b. Ice samples from the same core with an age of 30,000 yr (ice from the last glaciation) showed H_2O_2 levels at or below the detection limit of 0.5 p.p.b. During the cold periods of the last glaciation, the dust content in the ice was 10–100 times higher than during the Holocene¹¹. Perhaps dust was responsible for a catalytic destruction of the H_2O_2 during the snow formation. We cannot decide, however, whether these low concentrations are due to specific atmospheric conditions, or whether they reflect a slow decay of H_2O_2 in the natural ice sheet.

Our measurements show that chemically reactive species, such as H_2O_2 , are conserved in polar ice samples. The observed

concentrations are not surprising if one takes into account that measurements and model calculations predict similar mixing ratios in ambient air for HNO_3 and H_2O_2 (0.1–1 p.p.b.) and that both trace gases have comparable solubilities in water. If, therefore NO_3^- is found in ice cores, we can also expect H_2O_2 to be present. Although natural ice sheets seem to provide a record of the atmospheric chemical reactor, it is, however, not yet possible to establish any quantitative relationship between the H_2O_2 content of ice and that in the Arctic and Antarctic atmospheres. In the near future, we intend to investigate the effect of sample age on H_2O_2 concentrations to determine more precisely the interhemispheric differences.

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Age difference between polar ice and the air trapped in its bubbles

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Air entrapped in bubbles formed in cold ice has essentially the same composition as that of the atmosphere at the time of bubble formation. The analysis of dated ice samples therefore enables the history of atmospheric composition to be investigated.^{1–3} The age of the entrapped air is, however, not the same as that of the surrounding ice because air bubbles only become isolated from the atmosphere during the transition from firn to ice. Typically the age of the ice at this transition is between 100 and 3,000 yr, depending mainly on firn temperature and snow accumulation rate. The mean age difference between ice and enclosed air, as well as the age distribution width for a given sample, are especially important for the investigation of the anthropogenic increase of CO_2 and trace gases in the atmosphere over the last centuries, and for the comparison of climatic parameters recorded in the ice with parameters recorded in the bubbles. For Siple Station (Antarctica), this age difference and age distribution width were deduced from the bubble volume measured as a function of depth. The values are 95 yr and 22 yr respectively.

In polar regions, snow is compacted to firn, which is connected to the atmosphere through a system of interconnected pores. The thickness of the firn layer is typically 50–120 m, depending on the temperature and snow accumulation rate. At the depth

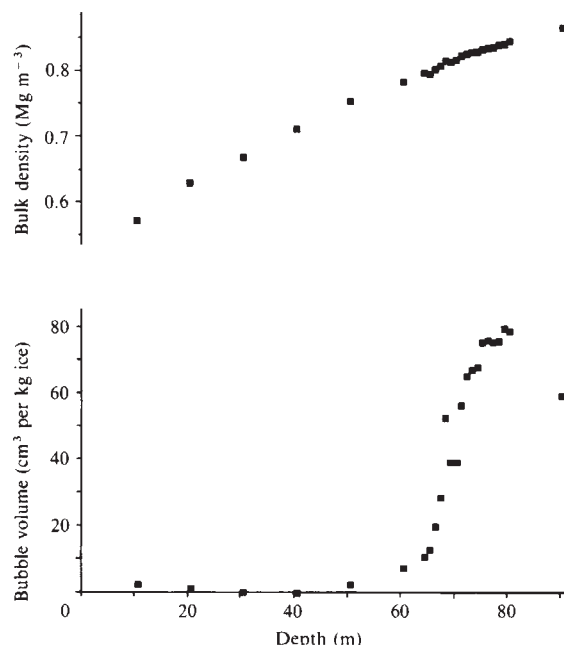


Fig. 1 Bulk density and bubble volume versus depth at Siple Station. Each point represents a 1 m average.

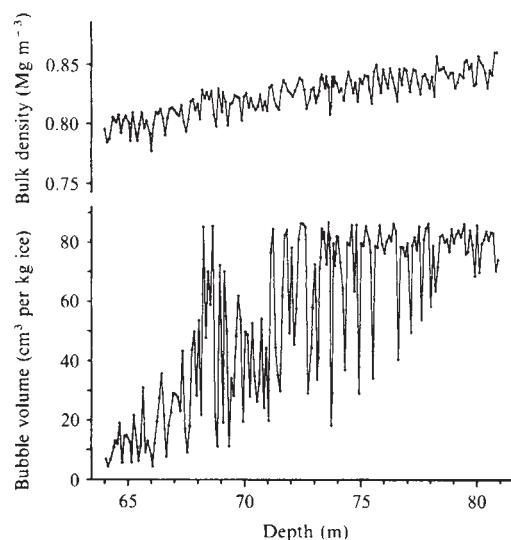


Fig. 2 Detailed bulk density and bubble volume profile of the Siple Station core at the firn-ice transition. Data points are connected by straight lines to illustrate their sequential order.

of the firn-ice transition, the pores are closed-off and individual bubbles are formed. The amount of enclosed air in firn as a function of depth has been measured previously by first removing and discarding the air from open pores, then melting the sample and measuring the amount of gas extracted during melting^{4,5}. Two methods have been used to remove the air from the open pores. In the first, the container is evacuated with the ice sample inside; however, this technique carries the risk that some air from closed, but still fragile, bubbles may also be extracted. For the other method, the air in the open pores is displaced by slow immersion of the sample. In this case, however, some of the air from open but dead-ended channels will not be removed.

To avoid the problems associated with removing the air first from open pores, we applied the classical method based on the law of Boyle-Mariotte for ideal gases: pressure $\times$ volume = constant at constant temperature. Samples are cut to a cylindrical shape of $\sim 35 \text{ cm}^3$. The bulk volume is measured with an

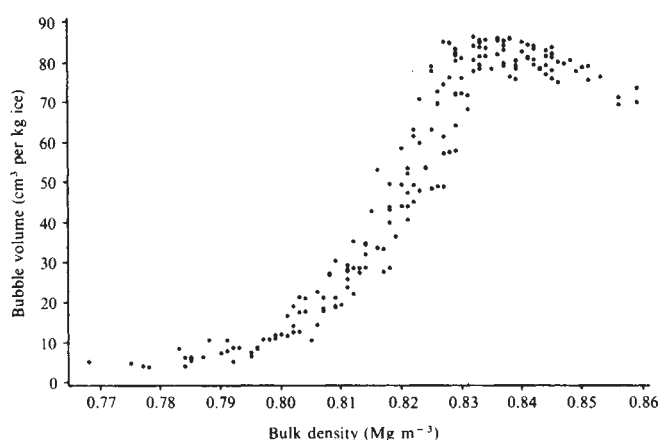


Fig. 3 Bubble volume versus bulk density from all samples between 60 and 81 m depth.

accuracy of 0.1%; the weight is measured with an absolute accuracy of 0.02 g. A calibrated cell containing the firn or ice sample at atmospheric pressure is isolated from the atmosphere and connected to a second, previously-evacuated cell. The pressure decrease, caused by the expansion at constant temperature, allows the volume of the closed bubbles to be calculated. We assumed a pure ice density of 0.919 Mg m^{-3} at -25°C . There is no danger of disrupting closed but fragile bubbles as the pressure drop is small (5,000–7,000 Pa). The accuracy of the density and bubble volume measurement is $\pm 0.002 \text{ Mg m}^{-3}$ and $\pm 2 \text{ cm}^3 \text{ kg}^{-1}$ respectively.

Measurements using this method were made on samples from an ice core drilled in November 1983 at Siple Station (75.92°S ; 83.92°W). The main objective for drilling this 192 m core was to determine quantitatively the increase in CO_2 content of the atmosphere since 1800 by analysing air extracted from samples at different depths. The core from Siple Station is especially well suited for this analysis because this location combines a low mean-annual air temperature (-24°C) with a rather high annual accumulation rate (0.5 Mg m^{-2}). Due to the low temperature, there is only a minimal risk, that the gas composition could be altered by meltwater⁶. A single meltlayer, of 10 mm thickness, was observed in the entire ice core. The high accumulation rate allows a good time resolution and the ice core was dated by counting the seasonal variations of the electrical conductivity of the ice⁷. The age error is $<2 \text{ yr}$ over the past 200 yr. CO_2 analyses on the Siple ice core will be carried out in the near future. We now focus on measurements which allow the age difference between ice and the enclosed air to be estimated.

All measurements on the ice core were done in a snow trench at South Pole Station⁸, where the temperature was very stable (-25°C). Density and bubble volume of 257 samples from 10 to 91 m depth below the 1982 snow surface were determined. Each point in Fig. 1 represents a 1-m section average, obtained from 10 measurements at 0.1-m intervals. Eighty per cent of the enclosure process takes place between the depths of 64 and 76 m. Only 10% of the final bubble volume is already enclosed above 64 m, and the remaining 10% is trapped below a depth of 76 m. Although at Siple Station (elevation 1,054 m) we expect a mean air content of the ice of $120 \text{ cm}^3 \text{ (STP) kg}^{-1}$ (ref. 5), the measured bubble volume is only $80 \text{ cm}^3 \text{ kg}^{-1}$ at the end of the pore close off (Fig. 1). We deduce a mean bubble pressure at this depth of 140 kPa. The pressure in the bubbles exceeds atmospheric pressure as soon as the bubbles are closed off due to the large local hydrostatic pressure. Detailed results for the depth interval 64–81 m are shown in Fig. 2. The cause of the variations in bubble volume and core density must be seasonal because these variations follow the pattern of the electrical conductivity measurements that distinguish between summer and winter deposits. The summer layers are characterized by low-density and low-bubble volume. The functional dependence between density and bubble volume is shown in Fig. 3 where only measurements between 60 m and 81 m are represented.

To calculate the mean age difference between the ice and the enclosed air, and the age distribution width, we have to assume an age of the air in the open pore volume at the transition zone which is connected to the atmosphere by many meters of permeable firn. ^{39}Ar measurements provide evidence that the air in the open pore volume is well mixed at least down to the beginning of the firn-ice transition⁹. Whether the air in the permeable layers sandwiched between almost impermeable layers is also well mixed with the atmosphere is uncertain. Assuming that the air is well mixed with the atmosphere in all permeable layers, then the mean difference between the age of the ice and the age of the air entrapped in its bubbles is about equal to the age of the ice at a depth where half of the final amount of air is closed off. For Siple Station the mean age difference obtained this way is 95 yr. The age distribution width, defined as the time interval during which the amount of entrapped air rises from 10 to 90% of the final value, is 22 yr. If impermeable layers such as the one from 72.0 to 72.5 m (Fig. 2) have a sealing effect, the mean age difference will be smaller, estimated to be $\sim 90 \text{ yr}$; the age distribution width should also be smaller, although it is difficult to estimate by how much.

Only 10% of the bubble volume is formed at a bulk density of $<0.795 \text{ Mg m}^{-3}$, while the final 10% is formed at densities above 0.83 Mg m^{-3} . Results from ice samples from Dye 3 (unpublished data) and North Central, Greenland¹⁰, suggest that these values might be valid for most polar sites. A semi-

Table 1 Measured values for Siple Station, and calculated values for other important sites, of the difference between the age of the ice and the mean age of the air trapped in its bubbles as well as the age distribution width

Site	Location	Mean annual air temperature ($^\circ\text{C}$)	Annual accumulation (m water equivalent)	Width of age distribution (yr)	Difference between mean age of ice and year (yr)
Siple	$75^\circ 55' \text{ S}$, $83^\circ 55' \text{ W}$	-24	0.5	Measured 22	95
				Calculated	
Vostok	$78^\circ 28' \text{ S}$, $106^\circ 48' \text{ E}$	-57	0.022	590	2,800
Dome C	$74^\circ 39' \text{ S}$, $124^\circ 10' \text{ E}$	-53	0.036	370	1,700
Southpole	$90^\circ 00' \text{ S}$	-51	0.084	220	950
Byrd Station	$79^\circ 59' \text{ S}$, $120^\circ 01' \text{ W}$	-28	0.16	54	240
North Central	$74^\circ 37' \text{ N}$, $39^\circ 36' \text{ W}$	-31.7	0.11	76	350
Crête	$71^\circ 07' \text{ N}$, $37^\circ 19' \text{ W}$	-30	0.265	46	200
Camp Century	$77^\circ 11' \text{ N}$, $61^\circ 09' \text{ W}$	-24	0.34	31	130
Dye 3	$65^\circ 11' \text{ N}$, $43^\circ 50' \text{ W}$	-19.6	0.5	22	90

empirical formula by Herron *et al.*¹¹ allows the density and age of the firn to be calculated as a function of depth if mean firn temperature, yearly accumulation rate, and snow density at the surface are known. We calculated the mean age difference between the enclosed air and ice, and the age distribution width using this formula for different stations, assuming that the air is of zero age in all open pores at any depth, and that 80% of the air is enclosed between the densities 0.795 and 0.83 Mg m⁻³. The results are given in Table 1. The width of the age distribution limits the time resolution for the detection of variations of the past atmospheric composition obtained from ice core analysis.

If the air is well mixed, even in permeable layers sandwiched between impermeable ones, then it should be of different ages in neighbouring seasonal layers. In periods showing an increase in the concentration of an atmospheric component we would expect summer layers to show higher concentrations than winter layers because bubbles in the winter layers close off earlier than those in the summer layers. Measurements of such a seasonal modulation of CO₂ concentration during a period of industrial CO₂ proliferation should provide detailed information on the CO₂ increase and the mixing of the air in the permeable layers at the firn-ice transition.

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Chronology of palaeoclimatic change at the end of the last glaciation

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Analysis of sediments recorded in a piston core, located east of southern North Island, New Zealand, gives a detailed record of climate change at the end of the last glacial. Accumulation rates¹ of both detrital (quartz) and biological (carbonate and biogenic silica) components of the sediment are much higher during glacial than post-glacial time and they all show a synchronous, rapid decline at 14,700 yr BP. We attribute this to a decline in intensity of the strong glacial westerly winds in the New Zealand region as the polar winds contracted to their present latitudes^{2–6}. The greatest rate of quartz accumulation occurs between 16,200 and 14,700 yr BP and is not matched by any change in carbonate or silica accumulation. We believe this reflects accelerated fluvial transport of detritus out of the mountain ranges and consequently increased aeolian dust transport before the decline of the polar westerlies.

Accumulation of greater amounts of quartz in deep-sea sediments during glacial periods than during times of warmer climate has been recognized^{7,8} and been attributed to increased wind erosion from arid regions during those cold periods^{7–11}. A marked change in global atmospheric circulation at the end of

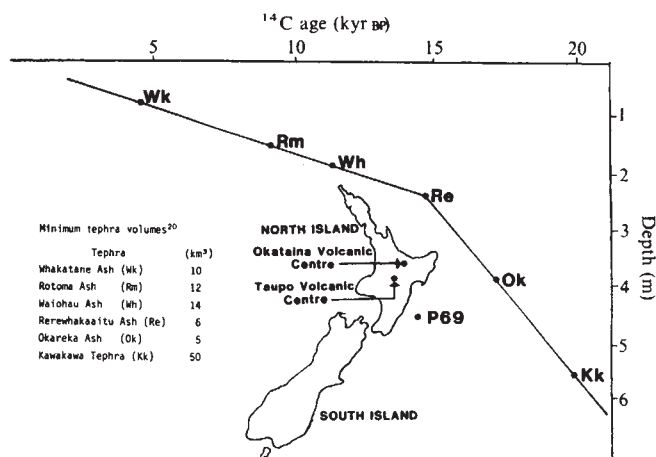


Fig. 1 Depth versus radiocarbon age of tephra in core P69 collected on cruise no 1059, Northland Plateau sediments, 17–31 March 1977 (from R/V *Tangaroa*), located at 40°23' S, 177°59.8' E. Mean pre- and post-Rerewhakaaitu Ash sedimentation rates are 594 mm kyr⁻¹ and 156 mm kyr⁻¹ respectively. Wk, Whakatane Ash (4,640 ± 90 yr BP, NZ 3949A); Rm, Rotoma Ash³⁷ (9,120 ± 30 yr BP, NZ 1945A) Wh, Waiohau Ash (11,250 ± 200 yr BP, NZ 568A); Re, Rerewhakaaitu Ash (14,700 ± 200 yr BP, NZ 716A); Ok, Okareka Ash (estimated age of ~17,000 yr BP from terrestrial sequences); Kk, Kawakawa Tephra (19,850 ± 310 yr BP, NZ 1056A). NZ numbers are New Zealand radiocarbon numbers. All tephra are from Okataina Volcanic Centre except Kawakawa Tephra, a Taupo Volcanic Centre eruptive.

the last glaciation has also been inferred from changes in manganese abundance in east-equatorial Pacific deep-sea sediments¹² and from changes in the microparticle content of polar ice^{13,14}. In the New Zealand region, differences in the distribution of quartz between surface sediments and those deposited ~18,000 yr BP have been used to infer that the westerly winds in the south-west Pacific were more intense and extended further north during the last glacial than they do today⁶.

As part of a wider project examining the accumulation of aerosolic dust in New Zealand we obtained a piston core from 2,195 m depth of hemipelagic sediment from a location 110 km east of southern North Island, New Zealand (Fig. 1). Quartz content in selected size fractions was determined by pyrolysis fusion and hydrofluoric acid digestion¹⁵, carbonate was determined by a titrimetric method¹⁶ and biogenic silica content by X-ray diffraction¹⁷. Time control is provided by six identifiable rhyolitic tephra^{18,19} erupted from the central North Island during the late Quaternary, five of which have been reliably radiocarbon dated in terrestrial sequences²⁰ (Fig. 1).

Rerewhakaaitu Ash (14,700 yr BP) occurs in a stratigraphical position marking major changes in sedimentation in the core. The mean sedimentation rate before this age is 594 mm kyr⁻¹, markedly greater than that of the younger sediment at 150 mm kyr⁻¹ (Fig. 1). As there is no evidence of major erosion or turbidities in the core, we believe that this change in sedimentation reflects the change in climatic conditions (especially a change in the westerly wind system) at the end of the last glaciation, influencing the supply of terrestrial sediment to the marine environment.

To elucidate the nature of this change in more detail the accumulation of three components of the sediment, one detrital (quartz) and two biological (carbonate and silica) were determined (Fig. 2). Quartz accumulation in both the 62–20 μm (representing loess) and 5–2 μm (representing aerosolic dust)²¹ size fractions showed a consistent, abrupt decrease in accumulation at ~14,700 yr BP. Quartz accumulation in both size fractions shows a maximum between ~16,200 and 14,700 yr BP. It is generally recognized that the glacial maximum occurred at about 18,000 yr BP in New Zealand²² and the period of maximum rate of quartz accumulation in the core clearly postdates this time. We interpret these data as showing that during and after the

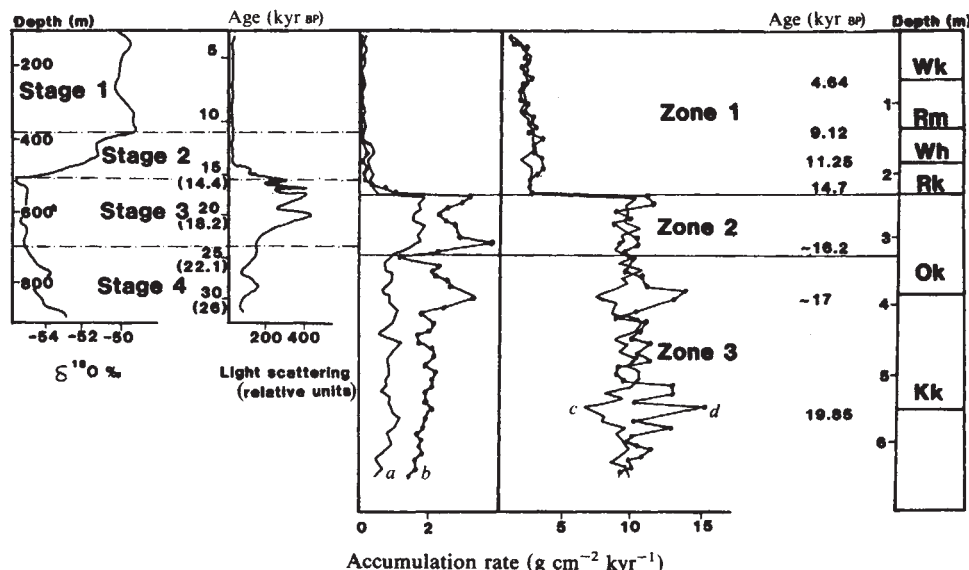


Fig. 2 Accumulation rates of: a, 5–2 μm quartz; b, 63–20 μm quartz; c, biogenic silica; d, carbonate. Tephra nomenclature is that described in Fig. 1. Zone 1 represents post-glacial sedimentation, zone 2 the period of accelerated erosion at the end of the last glaciation, and zone 3 the sedimentation during part of the last glaciation itself. The secondary maximum in 63–20- μm sized quartz is in part attributed to volcanic quartz from Okareka Ash, which occurs in this stratigraphical position. The oxygen isotope and microparticle (from light scattering in relative units) distribution curves are from Dome C ice core¹⁴ for comparison.

glacial maximum, a strong westerly wind system existed across the southern North Island. During the period 16,200–14,700 yr BP (zone 2, Fig. 1) we postulate an increase in fluvial activity in the early part of the climatic amelioration following the glacial maximum. This would increase transportation of erosion detritus stored in the mountain ranges during the cold, dry, glacial climate, to form the extensive aggradational surfaces of the lowlands. The continuing strong westerlies would have caused substantial wind erosion from these aggradational surfaces and resulted in increased aeolian transport of detritus to the marine environment east of the southern North Island. At ~14,700 yr BP the contracting westerly wind system passed to the south of the region and the abrupt decrease in wind erosion and transport resulted in a large decrease in the detrital component of sedimentation at this time (zone 1, Fig. 1).

The change in wind regime at 14,700 yr BP is also correlated with palynological evidence from central North Island where forest expansion began after this time^{23,24}. The change is also consistent with observations of the Rerewhakaaitu Ash overlying a widespread erosion break in terrestrial tephra sequences of central North Island^{23–25} and the cessation of tephric loess accumulation.

This interpretation of the quartz accumulation data is supported by the changes in accumulation of the biological components. These also show an abrupt decrease in accumulation rate at ~14,700 yr BP (Fig. 2), indicating a marked change in the marine biological environment at this time. Biological productivity in the oceans is a function of nutrient content, particularly phosphorus^{26,27}, of surface waters and nutrient concentration is generally higher in cold polar and sub-polar waters. Upwelling of these nutrient-rich waters is predominantly caused by strong winds^{27–31} and so patterns of biological detritus accumulation in the oceans are closely related to global wind patterns^{17,27,29}. The abrupt change in accumulation rate in biological components in the core at 14,700 yr BP is thus interpreted as a response to the strong glacial westerly wind system decreasing in intensity and contracting poleward at the end of the last glaciation. As the factors which caused increased accumulation of quartz between the glacial maximum and 14,700 yr BP would have had little effect on biological activity in the marine environment, no change occurred in the glacial accumulation rate of the biological components until the decline of the glacial westerly wind system ~14,700 yr BP.

Our data are consistent with a climatic amelioration beginning at ~16,200 yr BP, corresponding to initiation of a global rise in sea level^{32,33} following the glacial maximum. By ~14,700 yr BP the energy balance of the ocean–atmosphere system was such that the glacial circumpolar wind systems began to contract towards their present post-glacial latitudes. Evidence of a change

in particle transport at about this time has been found in both Arctic and Antarctic ice cores, concomitant with evidence of a change from cooler to warmer climate shown by oxygen isotope ratios of ice from the same cores^{13,14,34}. Comparison with the Dome C ice core¹⁴ shows that our zone 1/2 boundary correlates closely with their isotope stage 2/3 boundary and a similar distribution in microparticle content occurs (Fig. 2). Thus our inferred palaeoclimatic history from core P69 appears to be consistent with evidence from both deep-sea sediments and ice cores, suggesting that these changes were global. Finally, the terrestrial climatic changes reported here for New Zealand are for a maritime environment which may have responded more rapidly to global climatic variation than continentally glaciated areas on which ice sheets persisted into Holocene time^{35,36}.

This research is part of a wider study into the provenance of quartz in soils and sediments in New Zealand and we thank the New Zealand Department of Scientific and Industrial Research for funding.

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Turbulent motions may control phytoplankton photosynthesis in the upper ocean

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In their natural environment, algal cells may experience large amplitude variations in incident light on time scales shorter than those associated with the reproductive rate. A major source of variability is due to vertical mixing of water masses: turbulence in the upper mixing layer of the ocean transports algae through a light field that decreases exponentially from the sea surface. Several attempts have been made to discover how the physiological adaptation of algae to fluctuating light¹⁻⁹ might affect the magnitude of marine primary production^{10-16,17}, but it has proved difficult to estimate the vertical mixing rate at sea, and hence the time scales of irradiance variability^{18,19}. We recently succeeded, for the first time to our knowledge, in making direct measurement of rates of turbulent kinetic energy (TKE) dissipation, simultaneously with those of photoadaptation properties of natural phytoplankton populations. Dissipation of TKE in these experiments was a strong determinant of algal photosynthetic rate in the upper mixing layer; we conclude that the vertical mixing induced by turbulence to a large degree controls the photosynthetic performance of algae in nature.

The experiment attempted to test hypotheses generated by a recent theoretical analysis of algal photoadaptation in a mixing water column²⁰, where the significance of photoadaptation was evaluated by considering the time scales at which algal cells would be exposed to changing light intensity. As a first approximation, a simplified bulk view of the turbulent velocity field was taken. It is assumed that the algal cells remain uniformly distributed with depth. Then, any changes in a measurable index of photoadaptation (for example, maximum photosynthetic rate, *in vivo* fluorescence), at a particular depth, can be separated into two terms, one denoting physiological adaptation, and the other changes which result from turbulent fluid motion. As cells are transported in the vertical, irradiance incident on the cells varies. When the rate of photoadaptation is high relative to the rate of turbulence-induced light fluctuation, photoadaptation should produce variations in the photoadaptive property with depth. Conversely, when the rate of photoadaptation is low relative to that of turbulence, photadaptive variations in depth should be smoothed out by turbulence and consequently algal populations throughout the water column should be physiologically homogeneous. The physiological index, for a sample from a particular depth horizon, is an ensemble average of all cells present: it also contains (weighted) information about the recent light history of the cells. For fluid motions sufficiently rapid, samples taken anywhere in the mixing region should have the same average light history and therefore should be indistinguishable with respect to the magnitude of the physiological index. To test these hypotheses, a reliable and independent estimate of the rate of vertical mixing is required.

We therefore designed an experiment where the rate of turbulent mixing was determined simultaneously with the vertical distribution of photoadaptive indices. The experiment was carried out in Bedford Basin, a coastal marine inlet adjacent to the laboratory. Stations were occupied twice a day every 2-3 days for a one-month period during the spring. At each occupation samples were taken from the surface and bottom of a near surface mixing region distinguished from a temperature trace (Guildline SCTD) taken at time of sampling (Fig. 1). Maximum

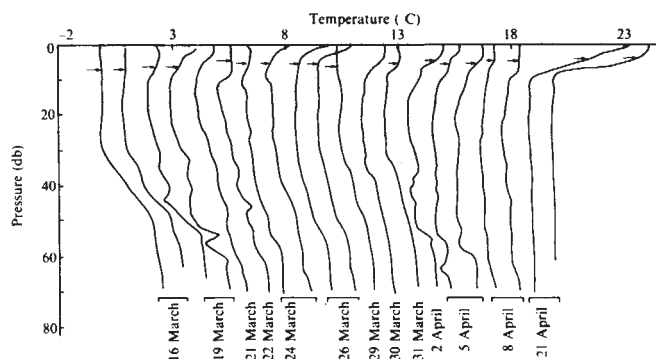


Fig. 1 Profiles of temperature during the study with the dates and sampling depth indicated. Profiles are offset by 1 °C. When two profiles are indicated for a single day, the first is a.m., the second is p.m.

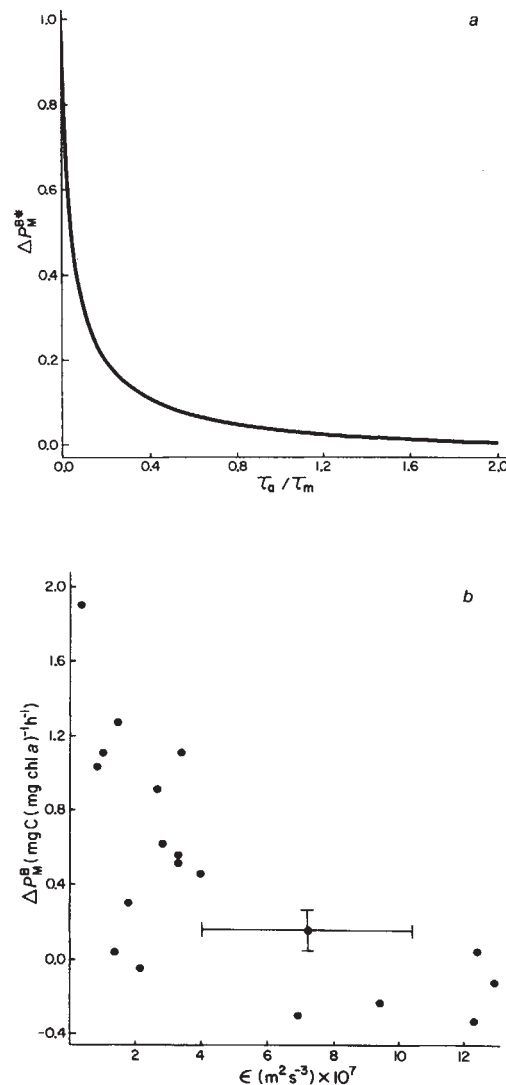


Fig. 2 a, Results from analytical solution²⁰. ΔP_m^B is the normalized difference between surface samples and those at the base of the mixing region. The irradiance gradient is the same as that in the field study. τ_a/τ_m is the ratio of the photoadaptation time scale to the mixing time scale. b, Measured differences between P_m^B at the surface and P_m^B at the base of the mixing region as a function of turbulent energy dissipation rate, ϵ . Error bars are ± 1 s.d.

potential photosynthetic rate normalized to chlorophyll *a*, P_m^B , was determined by methods described previously^{21,22}. *In vivo* fluorescence per unit chlorophyll, a photoadaptive index with a rapid response to irradiance changes^{23–25}, was also measured after a 15-min dark adaptation time. Rates of TKE dissipation, ϵ ($\text{m}^2 \text{s}^{-3}$), were determined from direct measurements of velocity shear microstructure^{26–29} from the recently-developed micro-scale profiler, Octuprobe²⁸ according to,

$$\epsilon = 7.5\nu \left(\frac{\partial u'}{\partial z} \right)^2 \quad (2)$$

where $(\partial u'/\partial z)^2$ is the averaged variance in turbulent velocity shear and ν is the kinematic viscosity. The numerical constant is a factor for isotropy. Values of ϵ over the depth interval 2–10 m were averaged over 6 or 7 realizations spanning 25 min. These values of ϵ were compared with differences between P_m^B and fluorescence parameters over the mixing region.

Results, shown in Fig. 2*b*, were consistent with the expectation of prior²⁰ theoretical analysis (Fig. 2*a*). At high TKE dissipation rates, little depth variation in P_m^B was observed; on days when energy dissipation rates were low, surface samples showed higher values of P_m^B (Fig. 2*b*). This strong inverse correlation between ΔP_m^B , which measures the physiological homogeneity of plankters over the mixing region, and ϵ , which is determined from dissipation scale fluctuations in velocity shear, does not result from an inconsistency of scales. The large-scale processes generating TKE may well be of the scale of the mixing region. Variations between days in the irradiance gradient were not significant. Order of magnitude mixing time scales to compare with those in Fig. 2*a* can be estimated from,

$$\tau_m = l^2 K_v^{-1} \approx 4l^2 \epsilon^{-1} N^2 \quad (3)$$

where l is the depth of the mixing layer, K_v is the eddy diffusivity and N is the Brunt–Väisälä frequency^{28,30–32}. Algal populations became physiologically more uniform throughout the mixing region as ϵ became larger. With $\epsilon = 4 \times 10^{-7} \text{ m}^2 \text{s}^{-3}$ and with measured values of $l \approx 5 \text{ m}$, and $N \approx 10^{-2} \text{ s}^{-1}$, the corresponding mixing time scale is $\approx 10 \text{ h}$. This value is consistent with laboratory estimates of the photoadaptation time of P_m^B ($= \tau_a$; Fig. 2*a*) of 6–10 h (refs 9, 20). Some of the observed residual scatter could be due to other environmental factors such as temperature, nutrient supply and grazing (see ref. 19). The relationship in Fig. 2*b* would suggest that the dominant source of variation responsible for the observed difference in physiological performance is turbulence-induced fluctuations in light incident on the algal cell.

Few motile cells were observed; the dominant species was the diatom *Melosira*. Populations of motile cells could potentially resist the vertical displacement induced by turbulence. If the swimming velocity is $\bar{w}$, a 'swimming time scale' can be estimated as $l\bar{w}^{-1}$ and compared with τ_m to determine which vertical motion would dominate. For a typical swimming velocity³³ of the order of 10^{-4} m s^{-1} , and the parameter values used here, ϵ would have to exceed $\approx 10^{-7} \text{ m}^2 \text{s}^{-3}$ for the mixing process to dominate, as it does here (Fig. 2*b*).

In-vivo fluorescence, when normalized to unit chlorophyll concentration, was less at the surface in the afternoon on all days except one—the day of highest measured dissipation. This was also consistent with our hypotheses; the time scale for variation in *in vivo*, chlorophyll-specific fluorescence on exposure to high light ($> 100 \text{ W m}^{-2}$) is $\approx 1 \text{ h}$ (refs 23–25), comparable with our highest estimate of $\tau_m \approx 1 \text{ h}$. The sea-surface irradiance (400–700 nm) varied between 100 and 400 W m^{-2} at noon. The observation of decreased fluorescence yield for most days at the surface is therefore consistent with a mixing time scale for these days greater than $\sim 1 \text{ h}$.

We conclude that turbulent fluid motion in the upper layer of the ocean controls to a large degree the physiological performance of algal cells. Only when energy dissipation rates are high can algal populations in the upper 'mixed' layer be considered physiologically homogeneous. It also appears from these data

that the rate of vertical mixing in the upper layer may be inferred from the vertical distributions of appropriate algal physiological indicators.

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Nitrate leaching from grassland

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Increased awareness of the economic importance of losses of nitrogen (N) from agricultural land, together with the recognition that nitrate in drinking water is potentially detrimental to human health, have stimulated research on nitrate leaching^{1–4}. Various reports^{5–7} have suggested that the leaching of nitrate from arable land is the major source of nitrate to water supplies in east and south-east England. In contrast, leaching from grassland is thought to be small^{5,8–10}. However, this conclusion has been based largely on studies of leaching below cut swards, which exclude the effect of ruminants (cattle and sheep) on nitrate loss. This effect is likely to be substantial as $< 20\%$ of the input of N to grassland systems is recovered in products of the ruminant animal¹¹. Our study demonstrates that the amount of nitrate leached below a grass sward grazed by cattle was 5.6 times greater than that leached below a comparable cut sward and exceeded the losses normally observed from arable land.

The extent of leaching was assessed by measuring the nitrate content of samples of soil or chalk removed in successive 0.33 m increments to a depth of 8 m below an experimental site at Hurley, Berkshire, UK. Management of the site is summarized in Table 1. The site was on a loam of the Frilsham series¹² overlying Upper Chalk which occurs at a variable depth between 0.7 and 1.3 m. Some properties of the top soil (0–20 cm) are:

Table 1 Management of the experimental site

Date	Plot 1 Cut ryegrass sward	Plot 2 Grazed ryegrass sward
January 1974– April 1976	Fodder maize* which received cattle slurry and farm-yard manure; annual input of N was estimated to be 450 kg ha ⁻¹	
April 1976–April 1977	Year during which the swards (each 0.123 ha) described below were established	
April 1977– December 1982	Cut perennial ryegrass† sward to which 420 kg N ha ⁻¹ as ammonia nitrate was applied annually. The sward was cut once every 4 weeks during the season April to October	Grazed perennial ryegrass† sward to which 420 kg N ha ⁻¹ as ammonium nitrate was applied annually. The sward was grazed rotationally (1 week in 4, April to October) by yearling steers. Average stocking rate was 9.3 head ha ⁻¹
December 1982– present	Swards ploughed and planted to spring barley‡ (April 1983) and winter wheat§ (September 1983). A total of 80 kg N ha ⁻¹ was applied in 1983	

* *Zea mays* L.; † *Lolium perenne* L.; ‡ *Triticum aestivum* L.; § *Hordeum vulgare* L.

pH, 6.0–6.5; bulk density, 1,250 kg m⁻³; organic carbon, 2.2–2.4%; total N, 0.25–0.28% (ref. 13). The bulk density of the sub-soil to a depth of ~1 m was 1,410 kg m⁻³ while that of chalk at depths >1 m ranged from 1,370 to 1,610 kg m⁻³ with a mean of 1,530 kg m⁻³ for 10 samples. The mean annual precipitation and potential evapotranspiration since 1974 were 694 and 500 mm, respectively^{14,15}.

The nitrate content of samples removed from below the grazed sward to a depth of ~6 m in December 1982, was consistently greater than that in samples below the cut sward to the same depth (Fig. 1a). Although Macduff and White¹⁶ have shown a log-normal distribution of nitrate content in soil, little evidence for this was found for the samples of chalk analysed in the present study. There were only small differences between the arithmetic and geometric means of nitrate content in the five replicates from each depth (Fig. 1 and Table 2). Consequently, a normal distribution was assumed during interpretation of the data obtained.

The differences between the mean nitrate contents of each profile to a depth of 6 m were significant at $P < 0.01$; the 95% confidence limits on each mean value were generally in the range ± 20 –30% (Table 2). The nitrate contents of samples from successive depths to 6 m within a particular profile were not significantly different ($P > 0.05$). At depths >6 m, the mean nitrate contents below the cut sward increased to values in the range 8.5–12.0 mg N kg⁻¹ and were not significantly different from those observed at the same depths below the grazed swards ($P > 0.05$).

Between April 1976 and December 1982, a total of 2,155 mm drainage was measured from lysimeters containing profiles of the Frilsham soil and installed within 500 m of the experimental site¹⁴. Based on a mean volumetric water content of 0.38 (95% confidence limits ± 0.011) in the samples removed and assuming simple piston flow, it is estimated that the 1976–77 leaching front associated with the first drainage season following establishment of the experimental swards should have reached a depth between 5.5 and 5.8 m. It was at about this depth that the nitrate content below the cut sward increased to values similar to those observed below the grazed sward. Thus, the similarity in nitrate content below both swards at depths >6 m apparently reflected the uniformity in management of the site before April 1976, when substantial amounts of waste from winter-housed cattle were applied (Table 1).

The average annual loss of nitrate from the experimental swards can be estimated by relating the mean nitrate content of samples from depths <5 m to the bulk density of material removed and to an estimate of the annual rate of water movement through the profile. Based on the above considerations, the rate of water movement between 1976 and 1982 was ~0.8 m yr⁻¹, a

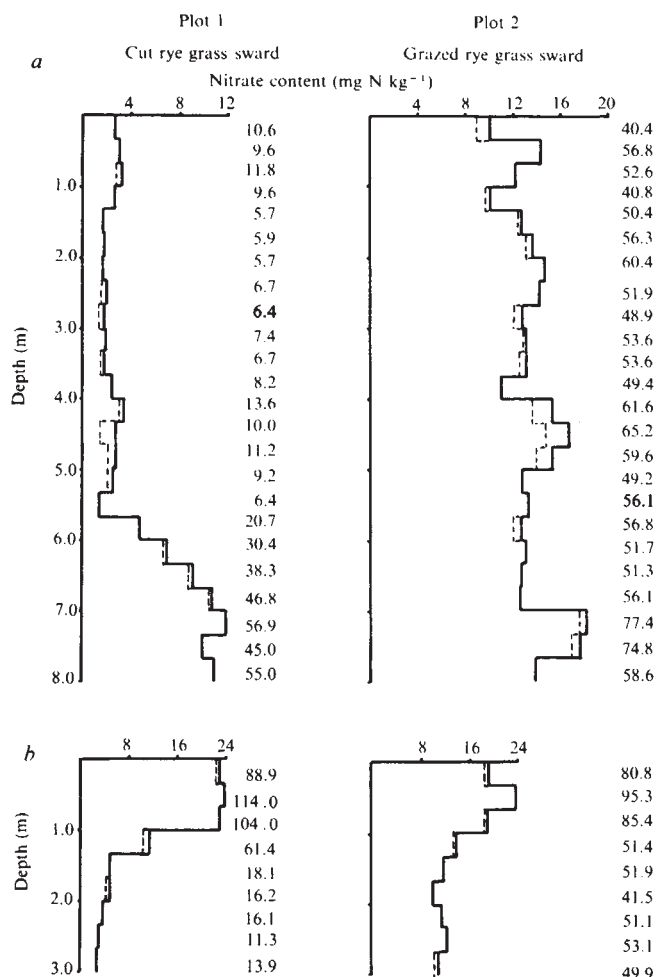


Fig. 1 Nitrate content (mg N kg⁻¹) of samples from successive depths below cut and grazed ryegrass swards (details in Table 1); a, before the swards were ploughed in December 1982; b, 12 months after ploughing and one crop of spring barley. The numbers alongside each profile are the nitrate concentrations in solution (mg N l⁻¹) at each depth. Values are the arithmetic means of five observations at each depth. The dashed lines indicate the geometric mean where this differed from the corresponding arithmetic mean by more than 0.3 mg N kg⁻¹. The mean values of nitrate content within the 0–1 m depth of each profile derived from the five replicates obtained in the present study were not significantly different from those derived from up to 39 samples removed from a similar depth in a preliminary study¹⁹. Samples for nitrate analysis were obtained using a power-driven flighted auger drill (Mobil Drilling Co., Indianapolis, USA) mounted on a Land Rover. Nitrate was extracted within 2 h of collection by blending 100-g sub-samples with 200 ml 2 M KCl. The supernatant solution was recovered after 2 h by filtration (Whatman No. 1) and its content of nitrate plus nitrite was determined using an automated colorimetric procedure based on the Griess-Ilosvay Cd-reduction method²¹. Nitrate comprised >99% of the nitrate plus nitrite content of extracts. The water content of samples was determined gravimetrically by drying in a forced air oven at 90 °C for 4 days.

value similar to others derived in studies at sites overlying chalk in south-east England^{6,10}. Hence, the average amounts of nitrate leached (nitrate content $\times$ bulk density $\times 8 \times 10^{-3}$) were 29 and 162 kg N ha⁻¹ yr⁻¹ for the cut and grazed swards, respectively. Nitrate leaching associated with the return of cattle slurry and farmyard manure during 1974 and 1975 was ~152 kg N ha⁻¹ yr⁻¹ based on the mean nitrate content of samples from below 6 m.

A substantial increase in the nitrate content of the upper 1.3 m of each profile was observed in December 1983, 12 months after

Table 2 Typical data for the nitrate content of samples from selected depth intervals within the profiles of nitrate content (Fig. 1) below cut and grazed swards

Depth interval (m)	Range	Arithmetic		Geometric mean
		mean	s.e.m. (mg N kg ⁻¹)	
Cut ryegrass		December 1982		
0.33–0.67	1.3–5.1	3.3	0.63	3.0
3.00–3.33	1.1–2.8	2.0	0.35	1.9
7.00–7.33	9.7–15.4	13.1	1.09	12.9
		December 1983		
0.33–0.67	21.8–25.9	24.1	1.71	24.0
2.33–2.67	2.8–3.6	3.2	0.29	3.3
Grazed ryegrass		December 1982		
1.00–1.33	9.1–17.4	12.6	1.68	12.2
3.67–4.00	8.8–14.7	10.6	1.06	10.5
7.33–7.67	10.2–20.2	17.2	1.81	16.7
		December 1983		
0.33–0.67	21.4–25.7	23.4	1.69	23.4
2.33–2.67	7.9–12.5	10.5	2.14	10.3

the experimental site was ploughed and cropped with spring barley (Fig. 1b; Table 1). Mean nitrate contents in the top metre of each profile ranged from 19.4 to 24.0 mg N kg⁻¹ dry soil, but differences within this depth and between profiles were not significant and did not reflect the differences in previous management. A discontinuity in the nitrate content of each profile occurred between 1.0 and 1.3 m, an observation in broad agreement with the estimated rate of water movement through the profiles. Although the nitrate front had spread into the upper part of the second metre, probably due to above average rainfall during spring 1983, nitrate contents below this depth were not significantly different from those measured in the corresponding profiles before ploughing (Fig. 1a). As 138 kg N ha⁻¹ were removed in harvested grain and straw in August 1983, the bulk densities and nitrate contents of the samples removed indicate that between 275 and 310 kg N ha⁻¹ in excess of crop requirements was mineralized and nitrified during the first 12 months following ploughing. A further 50 kg N ha⁻¹ was present as NH₄⁺ at the time of sampling. Much of this inorganic N is expected to be leached below 1 m during the 1983–84 drainage season.

The nitrate concentration in the water moving within each profile can be calculated from the nitrate contents (Fig. 1) and water contents (0.23–0.32 wt/wt) of the samples removed. Concentrations in water draining below the cut sward were in the

range 5.4–13.3 mg N l⁻¹, and were above the 'guide level' (5.7 mg N l⁻¹) but generally below the maximum (11.3 mg N l⁻¹) set by the European Community¹⁷ for water for human consumption. Concentrations of nitrate in water draining below: (1) the grazed sward; (2) the experimental area when slurry and farmyard manure were applied; and (3) both plots after they were ploughed, greatly exceeded the upper limit of the range 11.3–22.6 mg N l⁻¹ set by the World Health Organisation as 'acceptable'¹⁸.

The present study has shown that substantial amounts of nitrate can be leached from intensively farmed grassland as well as when grassland is ploughed in a ley-arable system. The most striking feature is the effect of ruminant production on nitrate leaching. The annual loss of nitrate from the grazed grass sward was 5.6 times greater than that below the comparable cut sward, despite a common input of fertilizer N and defoliation frequency. The enhanced nitrate movement below the grazed sward can be attributed mainly to the return in urine and dung of as much as 90% of the N in the herbage consumed by cattle¹¹. The effect of this is illustrated by the data in Table 3. At the end of the grazing season in October 1982, the mean nitrate content of soil below urine- and dung-affected areas of pasture was 3.2–7.7 times greater than that observed during random sampling across the grazed sward. Additional inorganic N was present as NH₄⁺ at this time, much of which had been nitrified by the time the profile was sampled in December at the onset of the drainage season (Fig. 1 and ref. 19). The small amount of residual inorganic N below the cut sward reflected the removal of at least 75% of the applied N in the harvested herbage. However, the cutting and conservation of herbage, and its subsequent use as feed, merely serves to delay the loss of nitrate that can occur after the disposal of slurry and farm-yard manure from winter-housed cattle (Fig. 1a and ref. 20).

The effect of ruminant production on the N cycle has received only limited attention in much of the recent work on the use of N in agricultural production^{1,5}. The present study and other reports^{21–23} show that this effect is substantial, particularly in intensive systems. The amount of fertilizer N applied to the swards used in the present study is typical of the amounts applied to intensively farmed grassland in north-west Europe^{24,25}. In England and Wales, 37% of the total area of grass leys now receives >200 kg N ha⁻¹ yr⁻¹, with 5% receiving >400 kg N ha⁻¹ (ref. 26); responses in animal production have been recorded for inputs up to 600 kg N ha⁻¹ (R. D. Baker, personal communication). The data indicate that as the management of grassland is intensified, the amounts of nitrate leached are likely to equal or exceed the range observed for continual arable production (40–100 kg N ha⁻¹ yr⁻¹, refs 10 and 27), the losses being greatly accentuated by ruminant production.

Table 3 Nitrate and ammonium contents of soil (0–30 cm) removed from below the cut and grazed swards at the end of the grazing season in October 1982

Sampling position	Number of samples	Nitrate content*				Ammonium content*			
		Arithmetic mean	s.e.m.	Geometric mean	s.e.m.†	Arithmetic mean	s.e.m.	Geometric mean	s.e.m.†
		(mg N kg ⁻¹ dry soil)				(mg N kg ⁻¹ dry soil)			
At random across cut sward	15	2.2	0.29	2.0	1.72–2.22	2.2	0.25	2.0	1.75–2.18
At random across grazed sward	15	7.5	2.23	5.6	4.64–6.63	4.4	2.23	2.2	1.69–2.83
Below urine-affected areas	8	53.5	14.79	43.8	37.11–51.7	56.3	12.56	41.7	33.25–52.25
Below dung-affected areas	8	26.2	8.22	17.8	12.58–25.23	15.1	3.76	10.4	7.09–15.18

* Although the arithmetic and geometric means of the nitrate and ammonium contents of soil below the grazed sward differed by a factor of 1.3–2.0, this was reduced to 1.1–1.2 when individual contents of each form of N were summed for each replicate sample and the mean values of total inorganic N calculated. Hence, the distribution of total N content in October was similar to that of nitrate in December by which time much of the ammonium had been nitrified (see Fig. 1 and ref. 19).

† Lower and upper values on the geometric mean.

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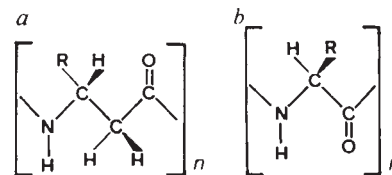


Fig. 1 Comparison of a segment of a nylon-3 derivative of the type described here (a) with a peptide unit in a protein (b). In a, $R = \text{COOCH}_2\text{CH}(\text{CH}_3)_2$. The lateral group R is on the opposite side to that in proteins, as it would be in polypeptide chains prepared from D-amino acids. The polymer was obtained by polycondensation of the pentachlorophenyl ester of α -isobutyl-L-aspartate hydrobromide according to Yuki *et al.*³ Its intrinsic viscosity is 0.57 dl g^{-1} in dichloroacetic acid at 25° which corresponds to a molecular weight of 90,000 if the equation for poly(γ -benzyl-L-glutamate) is applied⁶.

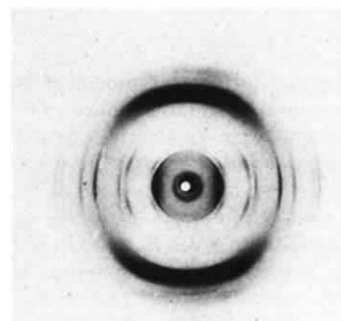


Fig. 2 X-ray diffraction pattern of a fibre of poly(α -isobutyl-L-aspartate) stretched from a chloroform solution. The stretching direction is on the meridian. Equatorial reflections correspond to an hexagonal unit cell with $a = 13.50 \text{ \AA}$. This crystallographic system provides additional evidence for a helical structure, because an extended structure should not pack in the hexagonal system. The first and fourth layer lines are clearly visible in the picture, with spacings of 19.9 and $4.98 \text{ \AA}$ respectively, which indicate a pitch for the helix of $4.98 \text{ \AA}$. The structure contains 13 monomer units in four turns of the helix.

A pseudo α -helix from poly(α -isobutyl-L-aspartate), a nylon-3 derivative

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Derivatives of nylon-3, with the general formula given in Fig. 1, crystallize as extended chains, producing sheets similar to those found in polypeptides in the β -conformation^{1–3}. Nylon-3 itself ($R = \text{H}$, poly- β -alanine) crystallizes as extended chains⁴. However, given the similarity of the chemical structure, we decided to search for conformations related to the α -helix in this type of compounds. We show here that the nylon-3 derivative poly(α -isobutyl-L-aspartate) can adopt a helical structure similar to an α -helix. The turn has 3.25 residues, equivalent to 13 main chain atoms, whereas the α -helix has 10.8 atoms per turn. This is the first time that a helical structure has been observed in a polyamide, other than in the conventional polypeptides and proteins.

Poly(α -isobutyl-L-aspartate) was prepared using the method described by Yuki *et al.*³. A stretched fibre of this compound gave the X-ray diffraction pattern shown in Fig. 2 which can be

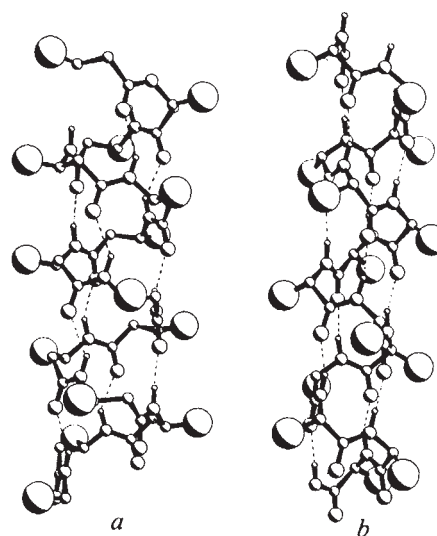


Fig. 3 Comparison between the left-handed helix observed in poly(α -isobutyl-L-aspartate) (a) and the conventional α -helix (b), constructed in this case with D-amino acids for a more adequate comparison. The larger spheres indicate the side chains. The smallest spheres correspond to the hydrogen atoms involved in hydrogen bonds, which are indicated as dotted lines. The rest of the hydrogen atoms are not shown. The model was built and refined using linked-atom-least-squares methodology⁷.

Table 1 Cylindrical atomic coordinates of a residue in the helical conformation of poly(α -isobutyl-L-aspartate)

z (Å)	r (Å)	ϕ (deg)	z (Å)
O	2.54	-35.8	0.81
C'	2.28	-29.6	1.99
N	2.14	3.4	2.39
H	2.11	11.1	3.35
C α	2.70	26.3	1.42
C β	2.72	59.1	1.59
C1 (R)	4.2	24.4	1.66
O1 (R)	5.06	27.1	0.83
O2 (R)	4.46	19.5	2.90
C2 (R)	5.60	9.6	2.90
C3 (R)	5.49	-5.6	2.46
C4 (R)	6.89	-8.0	1.87
C5 (R)	5.33	-15.5	3.68

Helix parameters: unit height $h = 1.53$ Å; unit twist = 110.8° ; pitch, 4.98 Å. r , ϕ and z are the cylindrical coordinates.

interpreted as a helix. The main argument in favour of such interpretation is that a weak layer line is observed with a spacing of 19.9 Å, together with a strong layer line at 4.98 Å. The first spacing corresponds to the true repeat of the structure, whereas the 4.98-Å spacing corresponds to the pitch of the helix. There should be a whole number of units in the 19.9-Å spacing. Model building indicates that there are 3.25 units in a complete turn of the helix and 13 units in the true repeat of four turns. Additional support for this interpretation comes from the hexagonal packing system found, which is typical of helical structures. The calculated density for such structure is 1.19, which is in reasonable agreement with the measured density of 1.17.

A model of the suggested structure was built and its coordinates are given in Table 1. It is shown in Fig. 3 together with the conventional α helix found in polypeptides. The following conclusions can be made from such a comparison: (1) The α helix has 10.8 main chain atoms per turn whereas the helix described here has 13. (2) The orientation of the hydrogen bonds is the same in both helices, so that they both have a polar structure. (3) To achieve such orientation, the nylon-3 derivatives maintain in the helix a conformation similar to that found in the extended chain, whereas in the α -helix there is a considerable rotation around the N-C and C-C bonds of the main chain so that consecutive peptide units have the same orientation. (4) The orientation of the side group with respect to the main chain in poly(α -isobutyl-L-aspartate) is similar to that found in a left-handed α -helix built from D-amino acids, in fact, our calculations show that there are fewer steric hindrances in a left-handed rather than a right-handed helix. (5) The average radius of the main chain atoms in the helix we have described is 2.4 Å, a value significantly larger than that found in the α -helix (1.8 Å), as it is apparent from Fig. 3.

Our studies show, therefore that it is possible to obtain conformations similar to the α -helix in nylon-3 derivatives. In fact, given the higher degree of flexibility provided by the additional carbon atom in the main chain, it is likely that other conformations (similar to either ω or π helices⁵) may be found in these compounds. To our knowledge it is the first time that a helical conformation has been found in a nylon derivative.

Replacement of oak forest with pine in the Himalaya affects the nitrogen cycle

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Pine and oak are the two predominant evergreen forests in the central and western Himalaya between altitudes 1,200 and 2,200 m. The pine is a light-demanding, fire-adapted but fire-promoting species. The surface fires averaging once every 2 or 3 years cause substantial nitrogen losses in pine forest. Pine forest naturally occurs on the driest and rockiest slopes, and has spread greatly under the influence of cutting and burning, replacing oak forest in vast areas. Oak forest is non-inflammable but has suffered a good deal from fire spreading from the pine forest¹. Deforestation has also accounted for loss of oak forest¹⁻³, and although the replacement of oak by pine is common^{1,2}, the reverse has not been observed. Our studies indicate that the greater nutrient-conserving ability of pine and the creation of a nitrogen shortage makes it difficult for oak to re-invade areas occupied by pine⁴⁻⁸.

The pine (*Pinus roxburghii* Sarg.) forest was studied on four sites and the oak (*Quercus leucotrichophora* A. Camus & Q. *floribunda* Lindl.) forest on three sites, between 1,300 and 2,100 m altitude, $29^\circ 24' - 29^\circ 37'$ N lat. and $79^\circ 28' - 79^\circ 38'$ E long. The annual rainfall is 1,160–2,298 mm, and mean annual temperature $13 - 18^\circ\text{C}$. All sites have mature (60–200-yr old) forests with uneven-aged trees. Soil in the pine forest is residue from chlorite-sericite schists and micaceous quartzites, weakly podzolic (pH 5.7), and sandy loam of composition 15.2% clay, 18.3% silt, 66.5% sand, 2.28% organic carbon and 0.18% total nitrogen. Oak forest soil is residual brown earth (pH 6.0), derived from limestone, quartzite and shales, and sandy loam (sand 76%, silt 13% and clay 11%) with 4.51% organic carbon and 0.51% N.

Biomass was estimated using allometric equations, and the density and diameter of trees. Net production was calculated from diameter increments and litter fall. One-fifth of the leaf fall was assumed to be equal to fine root mortality⁹, and this was added to the estimate of net production. All plant components were analysed for nitrogen and phosphorous. Gross uptake was calculated by multiplying the net production of different components by their respective nutrient concentration. The amounts of N and P transferred to the forest floor were estimated from litter fall and its nutrient concentration, and from canopy leaching. Standard error ranged between 3 and 6% of the mean values. The differences in nutrient concentration and dry weight of mature live and freshly senescent leaves were used to calculate the per cent withdrawal of N and P.

Dry matter, and the amounts of N and P for the two forests are given in Table 1. Dry weight of the standing vegetation in the oak forest was 1.8 times and N 3 times that of the pine forest. The amount of P was roughly similar. Soil (to a depth of 20 cm) under oak forest had 1.5 times as much N as in pine forest. The C:N ratio of oak soil was 8.5 and of pine soil 14.

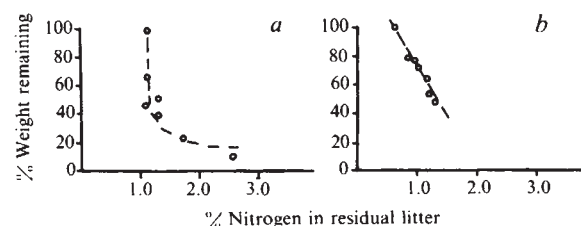


Fig. 1 Relationship between per cent weight remaining and N concentration of residual leaf litter after different time intervals during a year. *a*, Oak (*Q. leucotrichophora*) leaf litter enclosed in litter bags and placed on the forest floor of an oak forest; *b*, pine leaf litter enclosed in litter bag and placed on the forest floor of a pine forest.

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Table 1 Dry matter and N and P abundances for the tree layer of pine and oak forests

	Pine forest			Oak forest		
	Dry matter	N	P	Dry matter	N	P
Compartment pools (tonne ha ⁻¹)						
Vegetation						
Foliage	7.40	0.149	0.012	13.90	0.285	0.012
Branch	33.50	0.254	0.031	134.20*	1.571	0.065
Bole	131.10	0.656	0.092	153.40	0.966	0.046
Root	38.50	0.047	0.010	75.60	0.477	0.012
Total	210.50†	1.106	0.145	377.10	3.299	0.135
Litter	10.62	0.131	0.011	5.70	0.094	0.004
Soil (to 20 cm)	—	3.964	0.218‡	—	5.819	0.186‡
Total	221.12	5.201	0.374	382.80	9.212	0.325
Accumulation in net production (tonne ha ⁻¹ yr ⁻¹)						
Foliage	4.67	0.087	0.007	5.06	0.093	0.006
Branch	3.72	0.026	0.003	4.19	0.052	0.002
Bole	5.50	0.027	0.004	3.52	0.022	0.001
Root	2.81	0.003	0.006	2.33	0.018	0.0007
Total	16.70	0.143	0.920	15.10	0.185	0.010
Reabsorption before senescence (tonne ha ⁻¹ yr ⁻¹)						
Foliage	—	0.045	0.004	—	0.012	0.0005
Wood	—	0.008	0.001	—	0.006	0.0005
Total	—	0.053	0.005	—	0.018	0.0010
Net uptake from soil (tonne ha ⁻¹ yr ⁻¹)	—	0.091	0.010	—	0.167	0.009
Return (tonne ha ⁻¹ yr ⁻¹)						
Leaf litter	4.66	0.044	0.004	4.75	0.079	0.005
Wood litter§	2.05	0.010	0.001	1.38	0.014	0.001
Removal from canopy in through fall	—	0.004	0.0006	—	0.005	0.0004
Removal from canopy in stem flow	—	0.00006	0.00003	—	0.0001	0.0001
Root mortality	0.94	0.0009	0.0002	0.95	0.0076	0.0005
Total	7.65	0.060	0.006	7.08	0.107	0.007
Retention in vegetation compartments (tonne ha ⁻¹ yr ⁻¹)	9.05	0.031	0.004	8.02	0.060	0.002
Ratios						
Reabsorption: accumulation	—	0.37	0.33	—	0.10	0.11
Return: vegetation pool	0.04	0.05	0.04	0.02	0.03	0.06
Litter: litter fall	1.58	2.40	2.10	0.93	1.00	0.61

* Reproductive biomass was weighed with twigs and values are included in branch components.

† Cones in pine average 0.300 tonne dry wt ha⁻¹, 0.008 tonne N ha⁻¹ and 0.0007 tonne P ha⁻¹.

‡ Available P.

§ Includes reproductive litter.

Of the total N and P in the system, pine vegetation had, respectively, 22 and 40%, and oak vegetation 37 and 41%.

Net production was 8% of the tree layer biomass for the pine forest and 4% for the oak forest. Gross uptake of nitrogen was higher for the oak forest.

Substantial quantities of N and P were reabsorbed from old leaves before abscission in pine. Thus, 37% and 33% of gross uptake of N and P, respectively, were derived from nutrients reused from previous years in pine compared with 10% N and 11% P in oak. For foliage alone, 51% N and 52% P were reused for production of new leaves in pine, but 13.2% N and 8.5% P in oak. The withdrawal of nutrients before abscission maintains a relatively mobile pool within the living biomass of the ecosystem, and allows the plant to use the same unit of nutrient to build successively new leaves or plant parts^{10,11}. Increased internal redistribution of nutrients is considered to be an adaptation to impoverished soils¹²⁻¹⁵.

The net uptake of N and P from the soil is lower than the nutrient pool accumulated in net production (gross uptake) by the amount reused from previous years¹⁶. Net uptake in oak was relatively low for P, but for N it was considerably higher than in pine.

The return of N and P amounted to 3–6% of the quantities in the vegetation pool. The dry matter:nitrogen ratio of leaf litter fall was 105 in pine and 60 in oak. Thus the efficiency of litter production per unit of N was greater in pine compared

with oak. Vitousek¹¹ suggested that nitrogen efficiency of litter production varies as an inverse function of the nitrogen available.

The high, dry matter:nitrogen ratio of the litter and high, C:N ratio of the soil in pine forest reduce the rate of litter decomposition and increase the fuel load on the forest floor. In addition, the decomposers of litter of a high, dry matter:nitrogen ratio may immobilize available N from the soil solution. Weight loss and the N concentration of residual litter of pine and oak enclosed in litter bags and placed on respective forest floors were studied for 1 yr. Figure 1 clearly indicates a marked immobilization of N in decomposing pine litter. The dynamic available N pool in the soil at any point in time is usually only a small proportion of total N^{10,17} and its immobilization by microbes may intensify the N shortage in nitrogen-poor ecosystems. We suggest that this is the main reason why pine is able to resist reinvasion by oaks. As argued by Vitousek¹¹, low N availability caused by the immobilization by microbes may lead to increased efficiency of N use and production of litter with still higher dry matter:nitrogen ratio. Recurring fires in the pine forest lead to marked N losses through volatilization, augmenting the N shortage.

Oak has a relatively heavy demand for N and cannot succeed in N-poor soil. Through the return of N-rich litter and rapid mineralization, oaks maintain high soil fertility. Heavy logging of oaks for fodder and cutting for fuel cause large canopy gaps

and reduce leaf fall and hence N return, making the conditions suitable for invasion by pine.

The retention of N is specially pronounced in the oak forest (Table 1). Thus, in this forest the N pool may be more severely altered by deforestation. In such systems, replacement of N reserves will be essential to avert a long-term decline in productivity following deforestation¹⁰. In view of the above, the widespread replacement of mixed rain forests with plantations of conifers in the tropics and subtropics should be viewed with caution.

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Species variation and the mechanism of pressure-anaesthetic interactions

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The mechanism of general anaesthesia has proved difficult to elucidate (see ref. 1 for a review), although the relative potencies of anaesthetic agents have been used to establish that the site at which anaesthetics act is hydrophobic in nature². One further clue to their mode of action is that the effects of anaesthetics on vertebrates can be eliminated by pressures of ~100 atm (refs 3, 4). However, the effects of anaesthetics are not always reversed in model systems, where there is evidence that the pattern of pressure reversal varies significantly^{5–10}. We now find that pressure fails to reverse the effects of anaesthetics on the freshwater shrimp (*Gammarus pulex*), although the sensitivity of these crustaceans to anaesthetics is comparable with that of higher animals. This is hard to reconcile with traditional bio-physical mechanisms and indicates that anaesthetics may act at a specific protein site rather than having a general effect on cell membranes. The pharmacology of pressure in mammals seems to be more similar to that of strychnine than of any other central stimulant¹¹. As glycine, whose action is blocked by strychnine, is absent as a neurotransmitter in the arthropod central nervous system, we believe that this substance may be involved in determining pressure-anaesthetic interactions in vertebrates.

The effects of pressure and anaesthetics on the stimulated swimming activity of freshwater shrimps (*G. pulex*) and tadpoles (the larvae of *Rana temporaria*) were compared in similar experimental conditions. The animals were observed in glass cells, 5 cm long, designed to fit into a high-pressure chamber. The lower portion of the cells consisted of a trough of 1 cm² cross-section fitted with tungsten electrodes. Above the trough was a

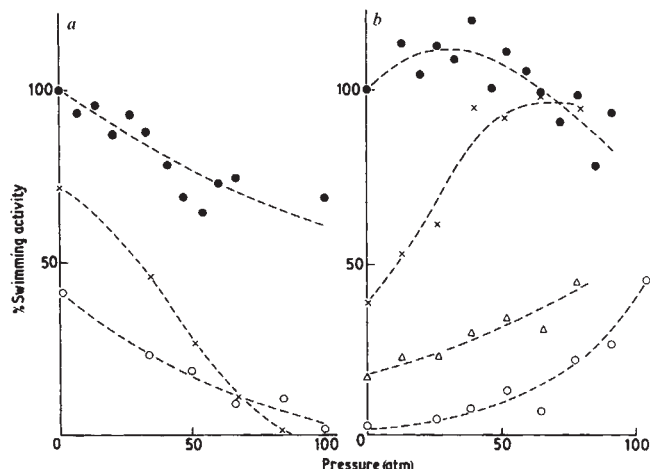


Fig. 1 a, Effect of pressure on *G. pulex* in the presence of ethanol (the lines are merely visual aids). b, Effect of pressure on larvae of *R. temporaria* in the presence of ethanol. ●, No anaesthetic; ×, 0.02 M ethanol; △, 0.265 M ethanol; ○, 0.275 M ethanol.

larger volume with steep sides so that anaesthetized animals fell into the trough which, when an electrical stimulus was applied, was a region not favoured by unanaesthetized animals. Five animals were placed in the cell and each determination of activity consisted of 20 observations taken at 10-s intervals. The fraction of the total population remaining above the trough was taken as a measure of swimming activity. Optimum swimming activity was obtained by the application of 5-V pulses of 0.1 s duration every 2 s for shrimps and similar pulses of 10 V for tadpoles. In these conditions the standard deviation of the measurements of activity was found to be $\pm 7\%$. The experiments were carried out at 18 °C. Anaesthetics were added directly to the cell and up to 30 min was allowed for equilibration. Pressure was applied using helium gas; this is an acceptable agent because any anaesthetic effects that it might induce are not significant at the pressures used here¹².

For shrimps, the relative potencies of the anaesthetics studied—ethanol, ether, chloroform, halothane and sodium pentobarbitone—were very similar to those determined with other organisms (Table 1). Furthermore, the absolute potencies for shrimps and tadpoles are close to those reported for other species. The effect of pressure, in the absence of anaesthetics, on the swimming of the shrimps was moderately to reduce activity by ~30% at 100 atm and no hyperactivity was observed (Fig. 1). With all the anaesthetics used, the application of pressure further reduced swimming activity. In no instance was there any evidence of pressure reversal; indeed, the decrease in activity seemed greater than would be predicted by the simple addition of the effects of pressure and anaesthesia. Tests were made to confirm that the effects of both pressure and the anaesthetics were reversible.

The experiments with tadpoles were carried out in a similar manner. Pressure alone caused moderate hyperexcitability. When anaesthetics were present, the application of pressure dramatically restored swimming activity (Fig. 2). This effect had been previously observed qualitatively with tadpoles¹³ and also with mice and newts³. Because tadpoles behave as expected in the same experimental conditions, it is unlikely that the anomalous behaviour of the shrimps is due to the experimental procedure.

This behaviour of the shrimps parallels that of the pleopod frequency of *Marinogrammarus marinus* which exhibits a gradual decline when exposed to 3.9 mm Hg pressure of halothane and which is potentiated by pressure³. For the brine shrimp, *Artemia salina*, the locomotor activity showed a lower relative decline on the application of pressure in the presence of anaesthetics than occurred in their absence. However, it was concluded that neither pressure reversal of anaesthesia nor a narcotic amelioration of the pressure effect had occurred¹⁴.

Table 1 Anaesthetic potencies

	<i>Gammarus</i> * (swimming activity)		<i>Goldfish</i> † (swimming activity)		<i>Mouse</i> ‡ (righting reflex)		<i>Tadpoles</i> § (swimming activity)	
	ED ₅₀	Ratio	ED ₅₀	Ratio	ED ₅₀	Ratio	ED ₅₀	Ratio
Chloroform	0.0008 ± 0.0002	1	0.0014	1	0.0011	1	0.0008	1
Ether	0.020 ± 0.003	25	0.029	21	0.20	18	0.024	30
Ethanol	0.25 ± 0.02	310					0.21 ± 0.02	260
Halothane	0.0004 ± 0.001	0.5	0.0038	0.3	0.00048	0.4		
Sodium pentobarbitone	0.0020 ± 0.003	2.5						

ED₅₀ doses expressed as molarities.

* This work; † ref. 23; ‡ data from ref. 24 corrected to molarity by method given in ref. 23; § ethanol; this work. Other data from ref. 25.

The relative potencies of the anaesthetics for shrimps, as for other organisms, point clearly to a hydrophobic site of action. The absolute potencies for shrimps and tadpoles are sufficiently close to suggest that in both species the anaesthetics act at physically similar sites by a common mechanism. However, the effects of pressure on anaesthesia in the two species suggests that very different mechanisms are involved. The effects of pressure on a process can be interpreted in terms of an associated volume change, ΔV (ref. 15). For a simple equilibrium, defined by equilibrium constant K ,

$$\frac{d \ln K}{dP} = \frac{\Delta V}{RT} \quad (1)$$

The association of ΔV with physical changes in the system depends on the model adopted¹. For vertebrates the volumes calculated from this equation, or obtained from more sophisticated models such as the constant volume hypothesis³, are positive and in the range 50–100 cm³ per mole of anaesthetic^{1,16}. This range is in keeping with the volume change associated with anaesthetic interaction at a hydrophobic region¹⁴. Conversely, the data for the shrimps, though not sufficiently precise to define quantitatively the associated volume change, indicate that the ΔV obtained from equation (1) is small and possibly negative.

Serious consideration has been given to two types of sites at which general anaesthetics might act. Traditionally, anaesthetics have been thought to act by a general perturbation of the lipid bilayers of the cellular membrane¹⁷. More recently, hydrophobic sites associated with protein molecules have been proposed¹. This idea has drawn support from investigations of the complex responses of bacterial luciferases to clinical concentrations of general anaesthetics¹⁸. Anaesthetics at low concentrations can enhance light production from the enzyme by facilitating substrate binding and at higher concentrations can decrease the light emission by blocking the binding of an aldehyde necessary for luminescence¹⁹. The effects are observed both *in vivo* and with the extracted enzyme. The reduction of light intensity caused by anaesthetics can be reversed by the application of pressure. The very different responses observed in shrimps and tadpoles are not easily explained in terms of general membrane sites, but the differences could be consistent with more specific binding at protein sites if, for shrimps, anaesthetics induce a conformational change in a protein which leads to a negative ΔV .

The pharmacology of pressure, as elucidated from studies of pressure-induced convulsions in mammals, has been found closely to parallel that of strychnine¹¹. This compound is believed to act primarily on postsynaptic inhibition, which is mediated by the neurotransmitter glycine. Crustaceans are insensitive to strychnine²⁰ and have a central nervous system in which glycine is not present as a neurotransmitter^{21,22}. Thus, we propose that pressure reversal of anaesthesia, as observed in vertebrates, may involve effects of pressure on strychnine-sensitive processes, possibly through changes brought about in the glycine receptor protein. Although we cannot rule out the possibility that pressure reversal results from an independent action of pressure leading to a physiological rather than pharmacological antagonism of anaesthesia, most models of pressure reversal assume that pressure and anaesthetics act at the same site^{1,4}. This suggests that in vertebrates, as in luminous bacteria, both

pressure and anaesthetics act on the same relevant protein. By analogy with the luciferase model, anaesthetics could facilitate the binding of glycine to its receptor protein, a process that could play a part in determining those aspects of general anaesthesia which are reversed by pressure, including loss of consciousness in mammals.

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Rules for neural development revealed by chimaeric sensory systems in crickets

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Many sensory systems are organized so that the afferent projection forms a topographic map of the sensory surface within the central nervous system (CNS)^{1,2}. The information necessary to create such a map may be available to the neuronal cell body based on its position in the receptor array, and this 'positional information' is translated into an axonal arborization in the proper part of the CNS^{3–6}. To study how the location of a cell body within the sensory surface determines the termination pattern of its axon within the CNS, we have transplanted epidermis, containing identified sensory neurones, from a black cricket to a tan cricket. As we report here, when epidermis is transplanted to an unusual location in the receptor array, newly generated neurones are produced along the borders of the graft. These neurones arborize in locations that are appropriate neither to their new position nor to their original position in the array, but rather to a position somewhere in between. This is direct evidence for the idea that positional information guides the differentiation and ultimately the synaptic connections of insect sensory neurones.

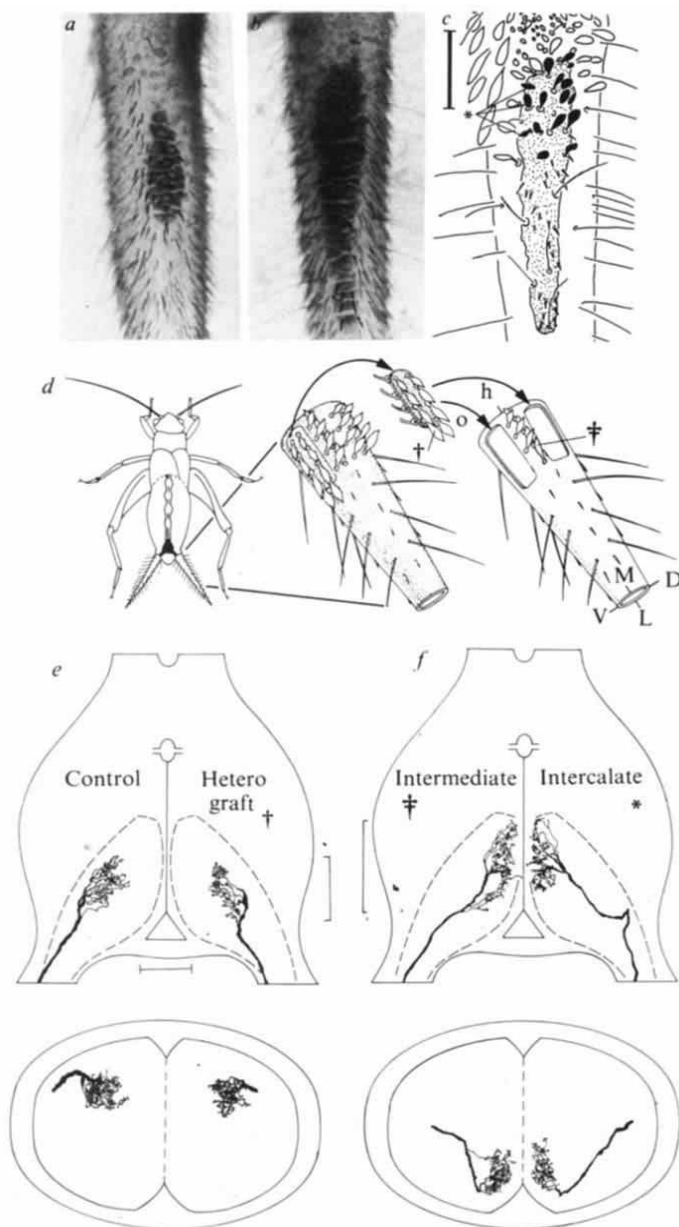


Fig. 1 Transplantation of neurones and characterization of their axonal arborizations. *a, b*, Light micrographs of chimaeric cerci. *a*, Ventral view of a cercus showing an orthotopic graft. The graft is schematized and indicated by 'o' in part *d*. *b*, Dorsal view of a cercus showing a heterotopic graft. This graft is schematized and indicated by 'h' in part *d*. *c*, Camera lucida drawing of the heterotopic graft shown in *b*. Transplanted clavate hairs (club-shaped) lie in the centre and to the right of the graft. *, Intercalated clavate hairs, as shown by their smaller size and by examining the graft for new hairs, after each moult. *a* and *c* are shown at the same magnification; scale bar, 500 μm . *d*, Schematic diagram illustrating the methods. The cutaway view of a cricket outlines the abdominal nervous system. The last abdominal ganglion, site of termination for all clavate neurones, is darkened. In each graft the two most ventral rows of clavate hairs and the surrounding epidermis were moved from a donor *G. bimaculatus* to a host *A. domesticus*. The graft consisted of a piece of tissue, 100 $\mu\text{m} \times 250 \mu\text{m}$, transplanted when both crickets were in the 6th instar. Phenyl thiocarbamide (Sigma grade II), penicillin G (Sigma) and streptomycin sulphate (Sigma) powders were combined by weight in a ratio of 2:1:1 and added directly to the wound to guard against infection and melanization. *e, f*, Comparison of the axonal arborizations of normal and transplanted neurones. *e*, The axonal arborization of a neurone transplanted to a foreign location (hetero-graft), indicated by † in *d*, is compared with the equivalent neurone on an unoperated cercus (control). *f*, The terminal arborization of an intercalated sensory neurone (intercalate) innervating a clavate hair along the ventral margin of the dark epidermis (not actually grafted but intercalated) (*c*, *) is compared with a neurone (intermediate) normally found between the new graft site and its site of origin (*d*, ‡). The upper panels in *e, f* represent dorsal views; the lower panels represent transverse views. The transverse views were reconstructed from serial sections (the region reconstructed is indicated by a bracket next to the dorsal view). The dashed lines in the dorsal views outline the target area, the cercal glomerulus, for clavate neurones. *e* and *f* are shown at the same magnification; scale bar, 100 μm .

An orderly array of club-shaped sensilla called clavate hairs, located on the cercus of crickets^{7,8}, forms the initial stage of a neuronal circuit that appraises the insect of its position with respect to the gravitational field⁹. Each clavate hair is innervated by a single epidermally derived sensory neurone whose axon projects into the CNS. As a group, these sensory neurones form a topographically ordered neuronal projection⁸. Analogous individual receptors can be identified on two species of cricket, *Acheta domesticus* and *Gryllus bimaculatus*. The only detectable difference is one of colour; *A. domesticus* epidermis is tan, whereas *G. bimaculatus* is black. To demonstrate the similarity of the two species, we transplanted strips of tissue containing the cuticle, underlying epidermis and receptors and associated sensory neurones from the cercus of a black cricket, the donor, to an analogous position (orthotopic graft, Fig. 1*d*, 'o') on the cercus of a tan cricket, the host. The transplanted tissue survived as a contiguous sheet of black epidermis; the borders between donor and host were sharply delineated and the hairs retained their colour differences (Fig. 1*a*), thus allowing donor-derived clavate hairs to be readily distinguished from their host-derived neighbours.

Because a stereotyped series of cell divisions produces a hair cell and its associated sensory neurone from a single epidermal cell¹⁰, a marked hair is always innervated by a sensory neurone

of the same genotype. Thus, *G. bimaculatus* epidermis provides a marker not only for cuticle, but also for the underlying sensory neurone. When identified donor neurones within the graft were stained, they possessed axonal arborizations indistinguishable in size and location from the equivalent host neurone on the undisturbed cercus (Table 1). Apparently, there are no species-specific differences between donor sensory neurones and the host CNS to prevent regeneration of normal axonal arborizations.

We have taken advantage of these similarities to study whether the position of the soma on the body surface determines the axonal arbor within the CNS. Donor sensory neurones were transplanted into non-equivalent areas of the cercus (heterotopic grafts), and they then regenerated arbors characteristic of their original position. For example, when ventro-medial donor tissue was transplanted to a dorso-medial location on the host (Fig. 1*d*, 'h'), the donor tissue healed into place and transplanted neurones terminated in a location within the CNS appropriate to their position of origin (Fig. 1*e*, hetero-graft). In fact, these heterotopic neurones are virtually indistinguishable from the equivalent neurone on the undisturbed cercus (Fig. 1*e*, control). Thus, some intrinsic property of the neurone allows it to regenerate an axonal arbor to its former location even after its soma has been transplanted to a foreign location.

How does a neurone obtain this instruction about where to terminate? If the position of origin of the soma is important in assigning the axonal arborization of a neurone to the proper target area, a translocation of the neurone or its precursor before differentiation (or determination) should assign it a new identity and lead to an axonal arbor in a location appropriate to its new position^{6,11}. Heterotopic grafts trigger the proliferation of new epidermal cells along the border between donor and host tissue¹²⁻¹⁴, as is suggested in Fig. 1a and b, which compare an orthotopic and a heterotopic graft: even though the donor tissues were initially the same size (0.025 mm²), the heterotopic graft grew significantly larger (0.343 mm²) than the orthotopic graft (0.139 mm², Table 1). It has been demonstrated, using external cuticular markers, that this new tissue represents positions normally located between the donor and the host and the epidermis is thus said to 'intercalate' the intermediate positions¹²⁻¹⁴.

When a heterotopic graft was created by transplanting a piece of donor epidermis containing identified clavate hairs to an unusual position on the host (Fig. 1d, 'h'), we noticed that new receptors often differentiated along the margins of the graft (Fig. 1c, asterisk). These new receptors could be identified by examining the graft after each moult; they were smaller and were not present at the time of the transplantation. Staining the axonal arbors of the sensory neurones associated with these intercalated receptors provides a critical test of the idea that sensory neurones are specified by positional information: if donor-derived neurones of 'intermediate' phenotype are produced, this supports the hypothesis that position of origin determines target area. When donor-derived neurones, associated with black hairs along the ventral border of the heterotopic graft (Fig. 1c, hairs indicated by an asterisk) were stained (Fig. 1f, 'Intercalate'), they projected to an area normally associated with neurones located in an area between that of the apposed host and the donor tissue (such as those innervating the hair indicated in Fig. 1d, ‡). Of 11 intercalated neurones stained in 11 different specimens, 10 could be recognized as having intermediate phenotypes (Table 1). Equally important, none of these neurones had the phenotype of the transplanted neurones (located more centrally in the graft, for example, Fig. 1e, heterograft). This result strongly supports the idea that relative position of the neuronal cell body, at or near the time of its terminal mitotic division, determines the shape and location of the neurone's axonal arborization in the CNS.

Bate^{6,15} suggested, from physiological determinations of neuronal connections, that the position of a sensory neurone on the epidermis of an insect regulates the central connections it makes. The present results and related work^{7,8,16} provide strong support for Bate's hypothesis because they show first that the axonal arborizations are physically distinguishable within the CNS, and second that the topography of the afferent projection is controlled by the position of origin of the cell body. The idea is very similar to Sperry's chemoaffinity hypothesis³, developed to explain results regarding the development of the projection from eye to brain in amphibians and fish. Subsequent work has led to considerable debate^{4,17-19} over the exact role of positional information in the formation of the retinal projection, primarily

because it is so difficult to identify and keep track of the neurones involved. In contrast, the individual sensory neurones of the cricket cercus, which are easily identifiable, have allowed us to demonstrate clearly that soma position is translated into a property that guides the elaboration of an axonal arborization in a particular location of the CNS. Because the location of the axonal arborization is ultimately associated with synaptic connectivity¹⁶, we conclude that positional information guides connectivity.

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Evidence for neurotensin as a non-adrenergic, non-cholinergic neurotransmitter in guinea pig ileum

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Neurotensin is a 13-amino acid peptide that is widely distributed in central and peripheral tissues of various mammalian species¹. In peripheral tissues, the highest concentration of neurotensin-like immunoreactivity is found in the ileum, where it is present in endocrine-like cells and nerve fibres²⁻⁴. The longitudinal smooth muscle layer of the guinea pig ileum, where neurotensin has both a direct relaxant and an indirect contractile action, has been used extensively as a biological assay system for neurotensin⁵. We report here that the majority of specific ³H-neurotensin binding sites is present in the guinea pig ileum circular smooth muscle layer, which is known to be innervated by a large proportion of the ileal non-adrenergic inhibitory nerves⁶. Neurotensin produces a dose-dependent, tetrodotoxin-resistant relaxation, whereas the relaxation produced by field stimulation of the inhibitory nerves is frequency-dependent and tetrodotoxin-sensitive. The calcium-dependent potassium channel blocker apamin^{7,8} inhibits both the neurotensin- and nerve stimulation-induced muscle relaxation. Incubation of the circular smooth muscle preparation with a neurotensin antiserum substantially inhibited the nerve stimulation-induced relaxation, indicating a direct relationship between the effects of neurotensin and of nerve stimulation.

For receptor autoradiography, adult male guinea pigs (250-400 g, Hartley strain) were stunned, a portion of their terminal ileum was dissected, flushed with cold saline, frozen and sectioned in the transverse plane at 13 µm using a cryostat. The sections were incubated with 2 nM ³H-neurotensin and non-specific binding was defined as that in the presence of 1 µM neurotensin. Following incubation, they were apposed to emulsion-coated coverslips and kept at -20 °C for 2 months. The

Table 1 Results of grafts between *A. domesticus* and *G. bimaculatus*

Type of graft	No. of successful grafts	Average size (mm ²)	Successfully stained neurones	Type of arbor
Orthotopic	11	0.139 ± 0.063 (n = 8)	5 Transplanted neurones	Normal
Heterotopic	30	0.343 ± 0.214 (n = 17)	6 Transplanted neurones 11 Intercalated neurones	Normal Intermediate*

The difference in average size between the two groups was significant at the 0.05 level ($t = 2.08$, d.f. = 22). Some of the chimaeric cerci were used for histological work and could not be included here.

* This arbor is located ventro-medially in the neuropil (see Fig. 1f, 'intermediate').

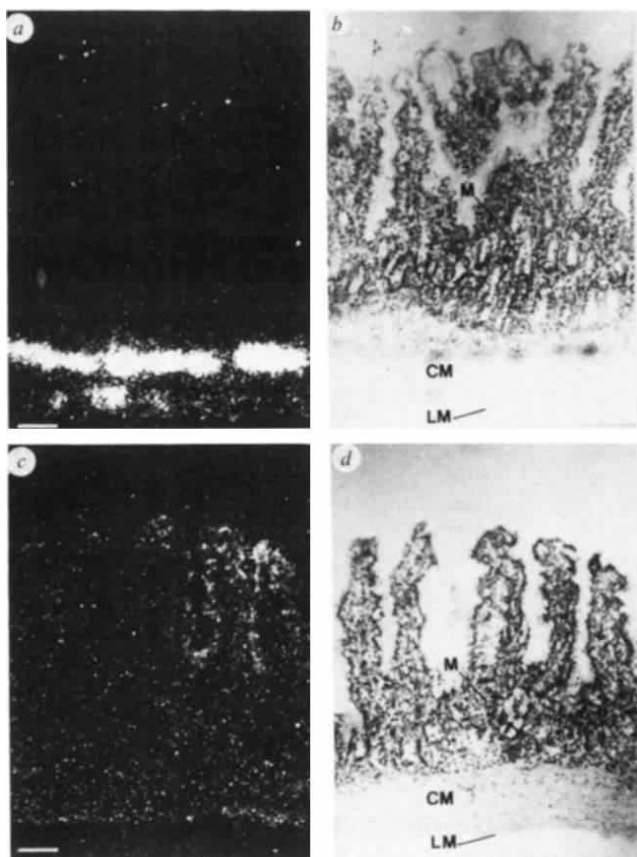


Fig. 1 Autoradiographic localization of ^3H -neurotensin binding sites in the guinea pig ileum. *a*, Dark-field photomicrograph of a section incubated in the presence of 2 nM ^3H -neurotensin. *b*, Light-field photomicrograph of the Nissl stain of the same section as in *a*. *c*, Dark-field photomicrograph of an adjacent 13- μm section to *a* incubated in the presence of 2 nM ^3H -neurotensin and 1 μM unlabelled neurotensin. *d*, Light-field photomicrograph of the Nissl stain of the same section as in *c*. M, mucosa; CM, circular muscle; LM, longitudinal muscle. Note the labelling of the circular and longitudinal muscle layers in *a*. Similar results were obtained for the ilea from six different animals. Scale bar, 100 μm .

Methods: Sections (13 μm) of the guinea pig ileum were pressed onto gelatin-coated slides, melted in place and dried overnight at 4 $^{\circ}\text{C}$ in air-tight boxes with desiccant. The thawed sections were incubated for 10 min at 25 $^{\circ}\text{C}$ in 2 nM [3,11-tyrosyl-3,5- ^3H]neurotensin (56.3 Ci mmol $^{-1}$, NEN) in 50 mM Tris-HCl pH 7.4, containing 0.1% bovine serum albumin (radioimmunoassay grade, Sigma), 40 mg l $^{-1}$ bacitracin (Sigma) and 1 mM EDTA. Nonspecific binding was defined in adjacent sections as binding in the presence of 1 μM unlabelled neurotensin (Cambridge Research Biochemicals). Following the incubation, the sections were washed twice in cold buffer (2 min each time), dipped into cold water and dried under a stream of cold air. They were then apposed to emulsion-coated coverslips²⁶ and kept at -20 $^{\circ}\text{C}$ for 2 months. The coverslips were developed in safe-light conditions using Kodak D19 developer and the tissue sections put into Carnoy's fixative, Nissl stained and mounted using Depex. Dark-field photomicrographs were taken of the silver grains and the Nissl stain, respectively. In some experiments, following the incubation with 2 nM ^3H -neurotensin, the dried tissue sections were scraped off the slides and the bound radioactivity determined by liquid scintillation spectrometry. The specific binding was 77 $\pm$ 9% (n = 3) of the total (taken as 100%). The binding of ^3H -neurotensin was not affected by 1 μM of the following peptides: neurotensin(1-6), neurotensin(10-13), substance P, vasoactive intestinal polypeptide, somatostatin(15-28) and dynorphin(1-17).

coverslips were then developed and the tissue sections Nissl-stained. Test-tube binding for ^3H -neurotensin was performed by using washed membranes prepared from whole guinea pig ileum. The guinea pig ileum circular smooth muscle preparation was set up as described previously⁹. Contraction of the muscle tone was induced by the tachykinin kassinin (10 nM) and all experiments were performed in the presence of atropine (5 μM), guanethidine (20 μM), indomethacin (1 μM) and mepyramine (5 μM).

When sections through the guinea pig ileum were incubated with ^3H -neurotensin, labelling was found in the smooth muscle layers, with a dense band of silver grains in the circular muscle layer and a band of lesser density in the longitudinal muscle layer (Fig. 1). The neurotensin binding sites were further characterized by using a test-tube binding assay for ^3H -neurotensin performed on washed membranes of whole guinea pig ileum.

The binding was saturable (Fig. 2*a*) and Scatchard transformation of the specific binding data (Fig. 2*b*) indicated the presence of a single population of binding sites with an equilibrium dissociation constant of 3.4 ± 0.7 nM (n = 4) and a maximum number of binding sites of 14 ± 2.6 fmol per mg protein (n = 4). The relative potencies of various neurotensin fragments in competing with ^3H -neurotensin for its specific binding site were similar to the potencies obtained for these fragments in the circular smooth muscle preparation (Table 1), indicating that the binding properties of ^3H -neurotensin were consistent with binding to a physiological receptor.

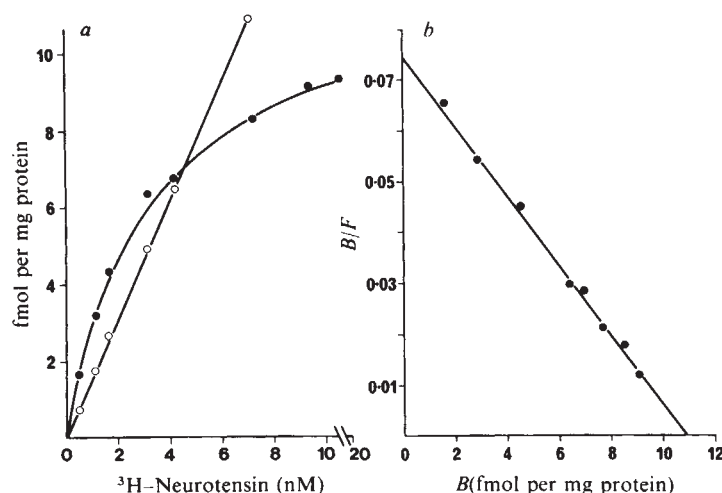


Fig. 2 ^3H -Neurotensin binding to a membrane preparation of guinea pig ileum. *a*, ^3H -Neurotensin as a function of the radiolabelled peptide concentration. Values were obtained in a single experiment; similar results were obtained in four separate experiments. Nonspecific binding ($\circ$) was defined as binding in the presence of 1 μM neurotensin and specific binding ($\bullet$) was obtained by subtracting the nonspecific from the total binding. *b*, Scatchard transformation of the specific binding data.

Methods: The ileum from adult male guinea pigs was dissected, flushed with cold saline, weighed and homogenized in 10 vol (w/v) of cold 50 mM Tris-HCl pH 7.4, with a Polytron at setting 7 for 15 s. After 20 min centrifugation at 50,000g, the pellet was resuspended in 10 vol 50 mM Tris-HCl, incubated at 37 $^{\circ}\text{C}$ for 30 min and centrifuged again. The washed pellet was resuspended in 50 mM Tris-HCl pH 7.4, containing 0.1% bovine serum albumin, 40 mg l $^{-1}$ bacitracin and 1 mM EDTA to yield a concentration of 10 mg tissue per ml buffer. 1 ml of homogenate was incubated in the presence of ^3H -neurotensin at 25 $^{\circ}\text{C}$ for 10 min. At the end of the incubation period the tubes were transferred to ice and filtered immediately under reduced pressure through GF/B glass fibre filters (Whatman) pretreated with 0.2% polyethyleneimine (Sigma) in water. Each of the filters was washed four times with 5 ml cold buffer and the radioactivity determined by liquid scintillation spectrometry. A detailed characterization of the binding assay has been published elsewhere²⁵. Proteins were determined according to Lowry *et al.*²⁷ using bovine serum albumin as the standard.

In the guinea pig ileum circular smooth muscle preparation, neurotensin produced a dose-dependent relaxation of the muscle tone, with an IC_{50} value of 3.6 ± 0.6 nM ($n = 12$, Fig. 3a), whereas the relaxation following field stimulation was frequency dependent (Fig. 3b). The latter was inhibited by 0.6μ M tetrodotoxin, whereas the muscle relaxation effected by neurotensin was tetrodotoxin-resistant. Both the neurotensin-mediated smooth muscle relaxation (1 – 30 nM) and the relaxation produced by inhibitory nerve stimulation (1 , 2 and 5 Hz) were completely inhibited by 30 nM of the bee venom apamin.

The possibility that the smooth muscle relaxation effected by inhibitory nerve stimulation is due to the release of a neurotensin-like substance was investigated by incubating the circular smooth muscle preparation with a previously described antiserum directed against the carboxy-terminus of neurotensin¹⁰. Incubation with the neurotensin antiserum (1 in 20 dilution) for 3 h not only prevented the action of neurotensin, but also inhibited the nerve stimulation-induced smooth muscle relaxation at all frequencies tested (Figs 3, 4). Higher dilutions of the antiserum at $1:50$ and $1:100$ were ineffective. Controls consisted of using the same dilution of either preimmune serum (Fig. 4) or an antiserum raised against [Lys⁸Asn⁹] neurotensin(8–13) a peptide sequenced from chicken small intestine which shares marked sequence homologies with neurotensin¹¹, but is not present in mammalian tissues¹². This antiserum, which was raised in the same way as the neurotensin antiserum and had a similar antibody titre, did not cross-react with synthetic neurotensin. Neither the preimmune serum (Fig. 4) nor the antiserum raised against [Lys⁸Asn⁹] neurotensin(8–13) affected the smooth muscle relaxation produced by either neurotensin or inhibitory nerve stimulation.

It was possible that the effect of the neurotensin antiserum was due to a non-selective postsynaptic action on the circular smooth muscle. However, the antiserum did not affect the contractile response to 10 nM kassinin (Fig. 4), nor the reduction

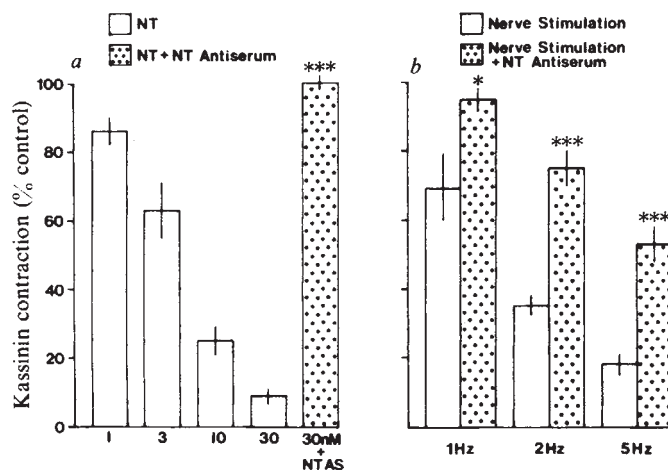


Fig. 3 Effect of neurotensin antiserum on the dose- and frequency-response curves to neurotensin (a) and non-adrenergic inhibitory nerve stimulation (b) in the guinea pig ileum circular smooth muscle. Note that the neurotensin antiserum completely reversed the inhibitory effect of neurotensin at all dose levels, with the effect on the maximum response of neurotensin (30 nM) shown above. The neurotensin antiserum also significantly antagonized the response to inhibitory nerve stimulation at all frequencies tested. All values have been expressed as a percentage of the control response to kassinin (100%). Open columns represent the controls and stippled columns the effects of neurotensin and inhibitory nerve stimulation in the presence of neurotensin antiserum. In each preparation, control responses were obtained before incubation of the tissues with the neurotensin antiserum ($1:20$ dilution) for 3 h at 37°C . Each value represents the mean $\pm$ s.e.m. of 4 – 10 observations. * $P < 0.05$; *** $P < 0.001$ (Student's t -test).

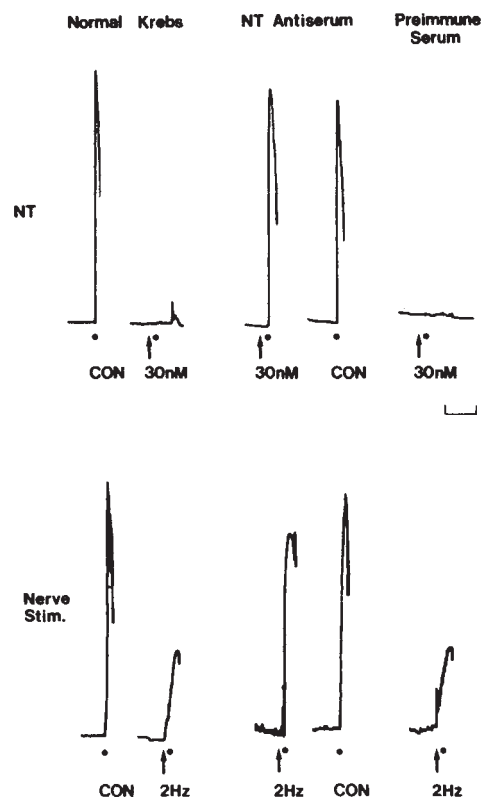


Fig. 4 Effect of neurotensin antiserum on the inhibitory responses of the guinea pig ileum circular smooth muscle to neurotensin and non-adrenergic inhibitory nerve stimulation. The upper row of records illustrates the effect of neurotensin (NT, 30 nM) and the lower row the effect of inhibitory nerve stimulation on the kassinin-induced tone of the guinea pig ileum circular smooth muscle preparation. Intramural nerve fibres were stimulated with 0.5 -ms pulses at supramaximal voltage and at a frequency of 2 Hz. The first pair of responses are in normal Krebs and represent, respectively, a control dose of 10 nM kassinin (CON) followed by a subsequent dose in the presence of either NT or nerve stimulation. The second pair of responses are in the presence of the neurotensin antiserum ($1:20$ dilution) following prior incubation of the tissues with the antiserum for 3 h at 37°C and the last response in each row shows the effect of prior incubation with preimmune serum ($1:20$ dilution) for 3 h at 37°C . Time bar, 2 min. Note that the neurotensin antiserum completely antagonized the inhibitory effect of neurotensin and substantially reversed the effect of inhibitory nerve stimulation, whereas the preimmune serum had no effect on either parameter.

Methods: Male guinea pigs (250 – 400 g, Hartley strain) were stunned, the terminal ileum was dissected, flushed with Krebs saline at 37°C and the circular smooth muscle preparation set up as previously described⁹. The tissues were suspended under a tension of 1 g in 2 ml organ baths containing Krebs saline at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2 . The saline composition was (in mM): NaCl 118 , KCl 4.7 , CaCl_2 2.5 , MgSO_4 1.2 , NaHCO_3 25 , KH_2PO_4 1.2 and glucose 11 . In all preparations tension was recorded with isotonic transducers. Contraction of the muscle tone was induced by 10 nM kassinin (CON) and all experiments were performed in the presence of atropine ($5 \mu\text{M}$), guanethidine ($20 \mu\text{M}$), indomethacin ($1 \mu\text{M}$) and mepyramine ($5 \mu\text{M}$). However, the contractile response to kassinin was found to be transient and hence inconvenient for the study of inhibitory responses in the conventional manner. The protocol of the present study was therefore similar to that of the anti-assay previously described by Burn²⁸. Briefly, neurotensin was added (arrow) 20 s (optimal) before the addition of kassinin ($\bullet$). Similarly, field stimulation of the inhibitory nerves was started (arrow) 20 s before the addition of kassinin ($\bullet$) and was continued until the peak of the response to kassinin was reached, normally 25 – 30 s. The direct inhibitory effects of neurotensin and inhibitory nerve stimulation on the circular smooth muscle have therefore been observed as a reduction in the contractile response to kassinin.

Table 1 Relative potencies of neurotensin fragments in a binding and a biological assay

Peptide	Binding assay		Biological assay	
	IC ₅₀ (nM)	Potency (%)	IC ₅₀ (nM)	Potency (%)
NT(1-13)	3.2 ± 0.5	100	3.3 ± 0.3	100
NT(8-13)	7.3 ± 0.7	43	11.5 ± 0.8	29
NT(9-13)	633 ± 90	0.50	344 ± 75	0.95
NT(10-13)	>10,000	<0.04	>10,000	<0.04
NT(1-6)	>10,000	<0.04	>10,000	<0.04

The binding assay was the inhibition of specific ³H-neurotensin binding to guinea pig ileum membranes and the biological assay the inhibition of kassinin-induced tone of the guinea pig ileum circular smooth muscle preparation. The binding of tritiated neurotensin (NT) was assayed at a concentration of 2 nM. The IC₅₀ was defined as the concentration of unlabelled peptide required to inhibit the specific binding of ³H-neurotensin by 50%. In the biological assay the IC₅₀ was defined as the concentration of peptide required to produce 50% inhibition of the contractile response to 10 nM kassinin. The relative potencies were calculated by comparing the IC₅₀ values with that of NT(1-13), taken as 100%. The results represent the means ± s.e.m. of between three and five separate experiments. The concentrations of NT(1-13) and of all NT fragments were determined by amino acid analysis. The purity of ³H-NT was determined by subjecting an aliquot from each batch to reverse-phase HPLC²⁵. NT(9-13) and NT(10-13) were synthesized by Dr B. J. Williams, NT(8-13) was purchased from Peninsula Laboratories and all other peptides were obtained from Cambridge Research Biochemicals.

(74.5 ± 5.3%, *n* = 4) in this response which was produced by the β-adrenoceptor agonist isoprenaline (500 nM).

The present results indicate that neurotensin has a potent direct relaxant effect on the guinea pig ileum circular smooth muscle layer, where a dense accumulation of specific ³H-neurotensin binding sites is found. The circular smooth muscle preparation may therefore constitute a favourable biological assay system for neurotensin. Electrophysiological studies have indicated that the circular smooth muscle layer of the guinea pig ileum is innervated by non-adrenergic, non-cholinergic inhibitory nerve fibres whose action is blocked by apamin^{13,14}. In addition, it has been shown that ATP and vasoactive intestinal polypeptide are unlikely mediators of this inhibitory non-adrenergic effect¹⁴. In the present study, the relaxant effects of nerve stimulation and neurotensin were both apamin-sensitive; a similar effect has previously been noted for the relaxant effect of neurotensin in the guinea pig colon and the rabbit ileum longitudinal smooth muscle^{15,16}.

This parallelism suggested that neurotensin may be released from non-adrenergic inhibitory nerve fibres following nerve stimulation. In the absence of neurotensin receptor antagonists, we have made use of a specific neurotensin antiserum to neutralize the endogenous peptide. Specific antisera have been used to study the physiological function of hypothalamic releasing hormones¹⁷⁻¹⁹; more recently, both polyclonal and monoclonal antibodies have also been successfully used to prevent the actions of neuropeptides in both the peripheral and the central nervous system²⁰⁻²⁴. In the present study, the neurotensin antiserum inhibited the nerve stimulation-induced smooth muscle relaxation to a significant extent, whereas both the preimmune serum and a control immune serum were without effect. The residual smooth muscle relaxation observed after exposure to the neurotensin antiserum may be due to the inability of the antiserum to neutralize rapidly all of the neurotensin released; alternatively, a substance distinct from neurotensin may contribute to the relaxation of the circular smooth muscle. In conclusion, the present results are consistent with the hypothesis that neurotensin functions as an inhibitory non-adrenergic, non-cholinergic neurotransmitter in the guinea pig ileum.

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Adenosine mediates a slow hyperpolarizing synaptic potential in autonomic neurones

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Considerable evidence has accumulated suggesting that purines, adenosine and ATP function as neurotransmitters in the central and peripheral nervous systems¹⁻⁴. Burnstock² has proposed that purinergic receptors be classified into two types, P₁ and P₂, having adenosine and ATP, respectively, as agonist prototypes. Recent data suggest that ATP may mediate a synaptic potential in guinea pig vas deferens^{5,6}, but no such evidence exists for adenosine. The presence of a non-cholinergic, non-adrenergic nerve supply to the urinary bladder has been postulated^{7,8} and termed purinergic^{1,2}, as these nerves have been shown to release ATP⁷. Furthermore, atropine-resistant contractions of bladder smooth muscle are thought to be mediated by ATP, while *in situ* experiments^{9,10} in vesical parasympathetic ganglia have suggested that a purinergic modulatory mechanism may control urinary bladder function. We now report the presence of a slow hyperpolarizing synaptic potential (slow-h.s.p.) in neurones of cat vesical parasympathetic ganglia, produced by stimulating the preganglionic nerves, and provide evidence that the slow-h.s.p. is mediated by adenosine.

Vesical parasympathetic ganglia isolated from the surface of the urinary bladder were placed in a chamber superfused with oxygenated (95% O₂/5% CO₂) Krebs' solution of the following composition (mM): NaCl, 117; KCl, 4.7; MgCl₂, 1.2; CaCl₂, 2.54; NaH₂PO₄, 1.19; NaHCO₃, 25 and glucose, 11. We employed conventional intracellular recording methods using glass microelectrodes (50-80 MΩ) filled with 2 M K₂SO₄ (ref. 11).

Vesical parasympathetic ganglion cells were superfused with a solution containing hexamethonium (1 mM) to block nicotinic responses to synaptically released acetylcholine in all experiments¹¹. Supramaximal stimulation (3-10 V) of the preganglionic nerve at 40 Hz for 250 ms in hexamethonium-containing solution revealed the presence of the slow-inhibitory postsynaptic potential (slow-i.p.s.p.), mediated through monosynaptic activation of muscarinic receptors¹¹ (Fig. 1a). When the stimulus

Fig. 1 Responses to nerve stimulation recorded in the presence of hexamethonium (C_6 ; 1 mM) and to exogenous application of adenosine (100 μ M) and ATP (50 μ M). *a*, Slow-i.p.s.p. elicited by preganglionic nerve stimulation (6 V, 40 Hz, 250 ms). *b*, Slow-i.p.s.p. recorded at a higher sensitivity in the same cell as in *a* after the stimulus intensity had been raised to 20 V; note the slower hyperpolarizing response on the falling phase of the slow-h.s.p. *c*, The slow-h.s.p., recorded from same cell as in *b*, remained after the slow-i.p.s.p. had been blocked with 1 μ M atropine. Note that the sensitivity of the recording was increased in *b* and *c*. *d*, *e*, Adenosine (100 μ M; *d*) and ATP (50 μ M; *e*) were applied by superfusion as indicated. Action potentials recorded in the ATP response were truncated. The downward deflections were produced by injecting constant-current anodal pulses of 100 ms and 1.4 nA (*a*), 0.7 nA (*b*, *c*) and 0.5 nA (*d*, *e*) through the recording electrode as a measure of membrane resistance.

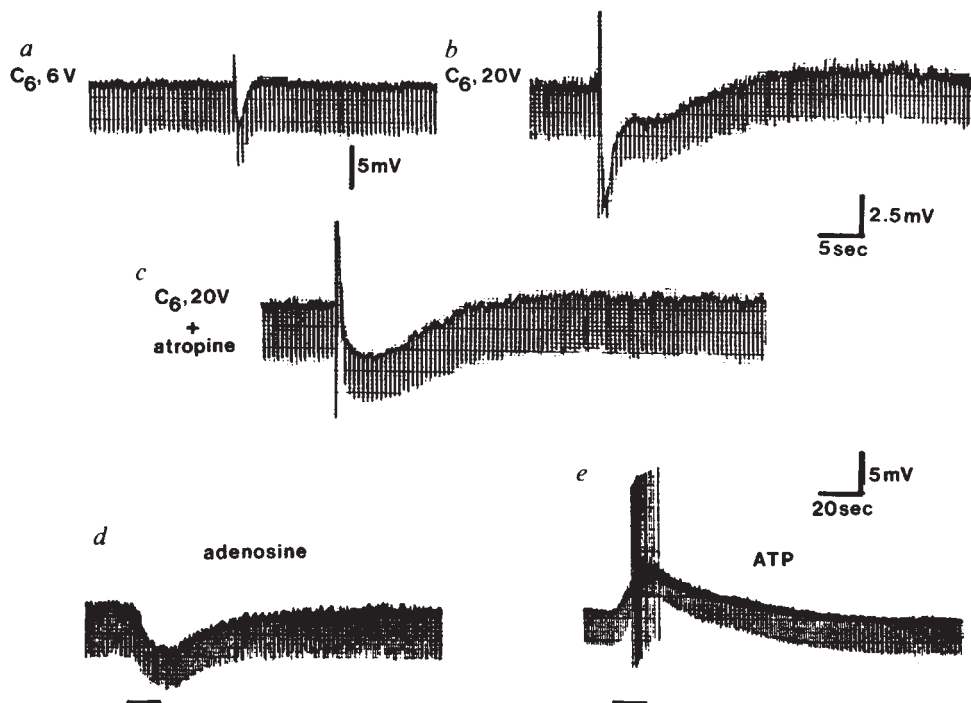
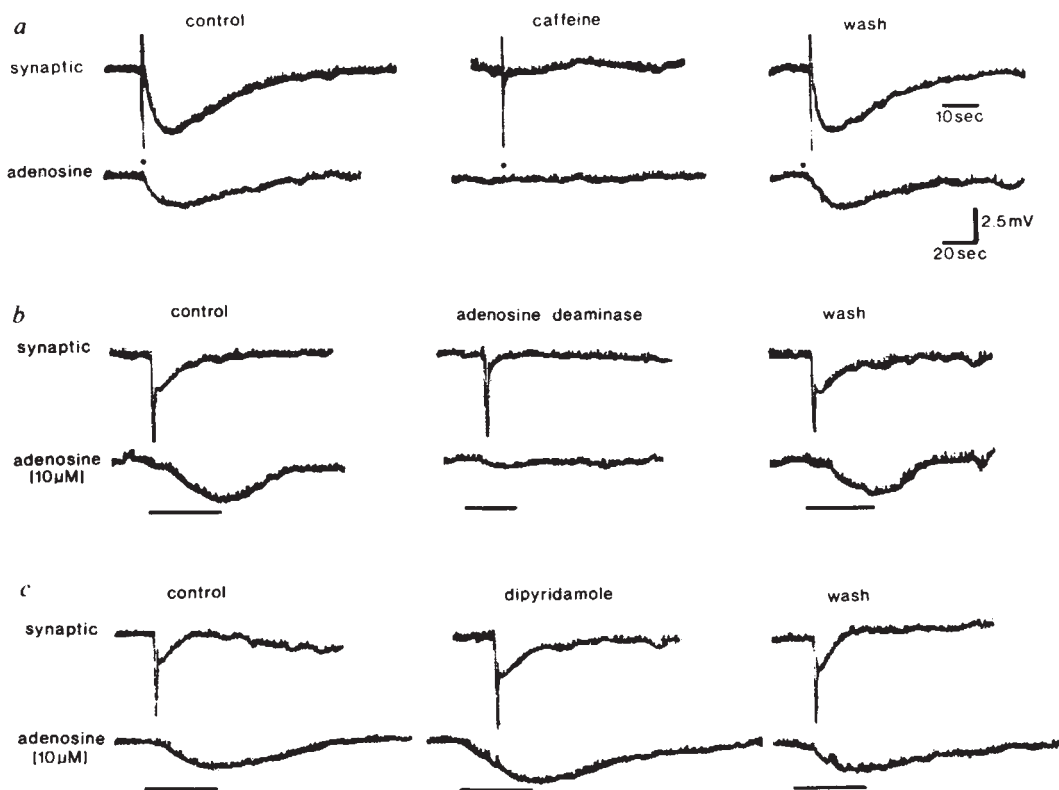


Fig. 2 Comparison of the pharmacology of the slow-h.s.p. and the adenosine hyperpolarization. *a*, The slow-h.s.p. and adenosine hyperpolarization in control conditions (left panels) and after 10 and 5 min treatment, respectively (middle panels), with 1 mM caffeine. The responses returned to control levels after a 15-min wash with normal Krebs' solution (right panels). Adenosine (100 μ M) was applied to the bath in a drop from an 18-gauge needle attached to a 1-ml glass syringe as indicated (dot). Resting potentials were -66 mV and -62 mV, respectively. *b*, The slow-h.s.p. and adenosine hyperpolarization in control (left panels) and 10 min after treatment with 0.25 IU ml⁻¹ adenosine deaminase (middle panels). The responses returned to control amplitude after 15 min washing with normal Krebs' solution (right panels). Resting potentials were -68 mV. Adenosine was applied by superfusion as indicated (bar). *c*, Slow-h.s.p. and adenosine response in control (left panels), 8 min after treatment with dipyridamole (1 μ M; middle panels) and 10 min after washing with control Krebs' solution (right panels). Resting potentials were -59 mV and -60 mV, respectively.

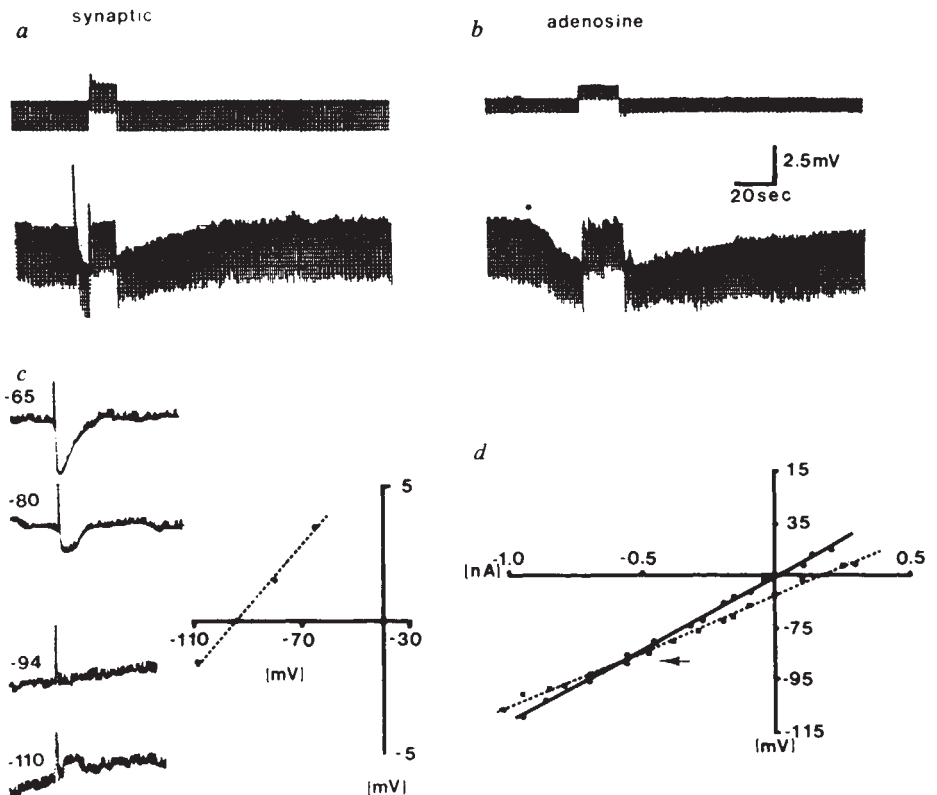


intensity was increased (8–30 V), a slower hyperpolarizing potential appeared on the falling phase of the slow-i.p.s.p. (Fig. 1b). Treatment with atropine (0.5–1 μ M) completely abolished the slow-i.p.s.p.¹¹ and revealed the presence of the slow-h.s.p. (Fig. 1c). This potential was quickly abolished in a low Ca^{2+} (0.25 mM), high Mg^{2+} (3.74 mM) solution, suggesting that the slow-h.s.p. was produced by a neurotransmitter released from the terminals of neurones whose axons were present in the preganglionic nerve trunk. The amplitude of the non-cholinergic slow-h.s.p. varied from 2 to 13 mV in individual neurones, with

a mean amplitude obtained from 58 neurones of 4.1 ± 1.7 mV (mean $\pm$ s.d.), recorded at a mean resting membrane potential of 62.3 ± 3.4 mV. The mean duration of the slow-h.s.p. was 31.6 ± 5.5 s, an order of magnitude greater than that of the muscarinic slow-i.p.s.p.¹¹.

Adenosine (5 μ M–1 mM) hyperpolarized (2–10 mV) most (92%) ganglion cells ($n=71$, Fig. 1d); other neurones (8%) were slightly depolarized by or did not respond to adenosine. The adenosine hyperpolarization persisted in low Ca^{2+} (0.25 mM), high Mg^{2+} (3.74 mM) solution for more than 25 min,

Fig. 3 Comparison of the membrane mechanism of the slow-h.s.p. and the adenosine hyperpolarization. *a, b*, Upper trace, the transmembrane current passed by means of a bridge circuit; lower trace, the membrane potential during the slow-h.s.p. (*a*) or during superfusion of adenosine (50 μ M) applied in a drop to the organ bath as indicated (dot). Resting potential = -68 mV in *a* and -60 mV in *b*. *c*, Left panel, slow-h.s.p. recorded at different membrane potentials in the presence of hexamethonium (1 mM) and atropine (1 μ M); right panel, graph of the relationship between the amplitude of the slow-h.s.p. (ordinate; 40 Hz, 250 ms) illustrated in left panels and membrane potential (abscissa). The slow-h.s.p. was nullified here, around -94 mV; the slow-h.s.p. reversed polarity with further hyperpolarization of the membrane. *d*, Hyperpolarizing and depolarizing current pulses of constant duration (100 ms) and varying amplitude were passed across the membrane in control solution (solid line; circles) and in the presence (dashed lines; squares) of a solution containing adenosine (50 μ M). The difference in the slope of the lines indicates a decrease in resistance during adenosine superfusion. The intersection point of the lines (arrow) represents the estimated reversal potential of the response, here -90 mV for adenosine. Lines were fitted by eye.



suggesting a direct action of adenosine on vesical neurones. The adenosine hyperpolarization also persisted in the presence of hexamethonium (1 mM) and atropine (0.5–1 μ M). On the other hand, bath application of ATP (100 nM–1 mM) produced a depolarization (3–12 mV) in 62% of vesical parasympathetic neurones tested ($n=63$, Fig. 1e). Some parasympathetic neurones (14%) showed a hyperpolarizing response to ATP (100 nM–1 mM) application, while others (24%) were not affected by ATP at these concentrations. In many cases, ATP and adenosine were applied to the same cells. These data suggested that the response to exogenous adenosine application is similar to the slow hyperpolarizing synaptic response.

Pharmacological analyses were performed to examine the possibility that the slow-h.s.p. is mediated by a purinergic neurotransmitter^{7,8}. Caffeine, known to be an antagonist of the P_1 purinoceptor², depressed the slow-h.s.p. recorded in 11 (73.3%) out of 15 neurones tested (Fig. 2a). The slow-h.s.p. was decreased $89.3 \pm 7.7\%$ ($n=8$) by caffeine (1 mM) in 5 min and abolished in 10 min. The effect of caffeine was concentration dependent, 100 and 10 μ M caffeine depressing the slow-h.s.p. by $64.4 \pm 7.1\%$ ($n=7$) and $41.8 \pm 5.2\%$ ($n=5$), respectively, after 5 min of treatment. In four neurones (26.7%), the slow-h.s.p. was relatively insensitive to the effect of caffeine (1 mM), being depressed by only ~22% after 10 min of treatment. Caffeine at these concentrations slightly hyperpolarized (1–3 mV) the membrane but did not significantly affect membrane resistance or directly evoked action potentials. Bath application of caffeine (1 mM) decreased the amplitude of the adenosine hyperpolarization by $92.6 \pm 4.3\%$ ($n=11$) after 5 min of treatment. The adenosine hyperpolarization was subsequently blocked reversibly by caffeine (Fig. 2a), but the response to ATP was not affected (not shown). These data suggest that the receptor underlying the synaptic response is similar to that mediating the response to adenosine—a P_1 purinoceptor.

Adenosine deaminase, the enzyme which catabolizes adenosine to inosine and NH_3 , depressed the amplitude of the slow-h.s.p. by $82.4 \pm 8.6\%$ (5 cells) after 5–10 min of treatment (Fig. 2b) and depressed the response to exogenous adenosine by $92.3 \pm 7.4\%$ ($n=7$). Bath application of adenosine deaminase

(0.25 IU ml^{-1}) produced no significant changes in the membrane potential or resistance. All the effects of this enzyme were reversible. The application of dipyridamole (3 μ M), which has been reported to block the uptake of adenosine¹² and augment adenosine effects in heart muscle¹³ and in neurones of sympathetic ganglia¹⁴, produced a slight hyperpolarization of parasympathetic neurones (2–4 mV), with no appreciable change in membrane resistance. At 1 μ M, dipyridamole increased the amplitude and duration of the slow-h.s.p. by $45.7 \pm 5.3\%$ ($n=6$) and $185.1 \pm 0.3\%$ ($n=6$), respectively (Fig. 2c), while the amplitude and duration of the response to exogenous adenosine were enhanced by $128.3 \pm 10.3\%$ ($n=6$) and $136.6 \pm 15.4\%$ ($n=6$), respectively. These data suggest that the synaptic response, the slow-h.s.p., and adenosine hyperpolarization are affected by pharmacological agents in a similar manner.

We compared the membrane mechanism of the slow-h.s.p. with that of the adenosine hyperpolarization (Fig. 3). The slow-h.s.p. was associated with a decrease in membrane resistance (Fig. 3a). The changes in membrane resistance could be separated from membrane rectification by passing a cathodal current through the recording electrodes and returning the membrane to its original resting level during the peak of the slow-h.s.p. or adenosine hyperpolarization. The mean decrease in resistance during the slow-h.s.p. was $35.8 \pm 8.8\%$ ($n=12$). Adenosine (100 μ M) induced hyperpolarization was also associated with a decrease in membrane resistance (Fig. 3b), to $33.7 \pm 5.6\%$ ($n=9$) of that at original resting potential levels. We also compared the reversal potential for the synaptic response with that of the adenosine hyperpolarization. The amplitude of the slow-h.s.p. was decreased by membrane hyperpolarization and eventually reversed in polarity at a membrane potential of -95 mV (Fig. 3c). The mean reversal potential of the slow-h.s.p. obtained in five neurones was -93.7 ± 7.4 mV. As Fig. 3d shows, the slope of the current-voltage ($I-V$) curve was decreased in the presence of adenosine (100 μ M). The reversal potential of the adenosine hyperpolarization estimated by the intersection of $I-V$ curves determined in control and adenosine (50 μ M) containing Krebs' solution¹⁵ was -90 mV; the mean reversal potential of the adenosine hyperpolarization obtained from nine neurones was

-93.5 ± 4.3 mV. The amplitude of the adenosine hyperpolarization was increased in K^+ -free solution to $175.5 \pm 10.3\%$ of the control response ($n=9$). The slow-h.s.p. was also augmented in a low- K^+ (0.5 mM) solution, to $156.1 \pm 8.1\%$ of the control value ($n=7$). Neither the slow-h.s.p. nor the adenosine hyperpolarization was affected by prolonged injection of Cl^- ion, applied by passage of outward current through an electrode filled with 2 M KCl. This indicates that Cl^- ions are probably not involved in the responses. These data suggest that both the slow-h.s.p. and the adenosine hyperpolarization are mediated primarily through activation of a K^+ conductance. A similar ionic mechanism for a purinergic hyperpolarization has been reported in guinea pig taenia coli¹⁶, frog heart muscle¹³ and *Xenopus* oocyte¹⁷.

Our data demonstrate that a synaptic response, a slow-h.s.p., produced by preganglionic nerve stimulation in vesical parasympathetic neurones in the presence of cholinergic blocking agents, can be most closely mimicked by exogenous application of adenosine. The present results seem to satisfy the pharmacological and electrophysiological criteria for establishing adenosine as a neurotransmitter in vesical parasympathetic ganglia. These data provide the first evidence for a synaptic response mediated by adenosine and lend further support to the mounting evidence that purines function as neurotransmitters in the autonomic nervous system.

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Partial correction of murine hereditary growth disorder by germ-line incorporation of a new gene

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The dwarf little (*lit*) mouse is a model for the human hereditary disorder, isolated growth hormone (GH) deficiency type I¹. In these animals, dwarfism results from an autosomal recessively inherited gene mutation. The *GH* gene is present² but production of *GH* mRNA is deficient³, resulting in reduced serum GH and concomitantly decreased serum somatomedin⁴⁻⁶. Growth retardation is evident by 15 days of age and adult animals reach approximately one-half normal size¹. Mutant mice of both sexes also exhibit a delayed onset of puberty, with males having a high degree of

infertility¹. As administration of GH restores growth⁷, we reasoned that growth failure in the mutant mice might be corrected by providing them with sufficient GH by gene therapy. Here we demonstrate that although the rat and human *GH* genes alone do not restore growth in transgenic mutants, a metallothionein-rat growth hormone fusion gene (*MT-rGH*) does. Moreover, the fertility of transgenic mutant males is improved; however, female fertility is impaired.

As one approach to gene therapy, we tested the feasibility of introducing either rat or human *GH* genes that contained normal 5' and 3'-flanking sequences^{8,9}. Because mutant eggs are difficult to obtain, we piloted this approach with normal mice. Thirty-two transgenic mice carrying one or more copies of the foreign *GH* genes were produced, and despite low levels of human growth hormone (*hGH*) mRNA transcripts in the liver and pituitary of some mice carrying the *hGH_N* and *cGH4* genes (data not shown), no foreign GH was detectable and none of the mice grew larger than normal (Table 1). This lack of *hGH* gene expression has occurred in other *hGH* mice¹⁰ and may reflect a requirement for precise chromosomal localization^{11,12}, insufficient flanking sequences or highly species-specific regulation of *GH* gene expression. The absence of detectable serum *hGH* and increased growth suggested that introducing normal *GH* genes into mutant eggs would be ineffective; therefore, we attempted to correct the growth disorder of mutant *lit/lit* animals by introducing a *MT-rGH* fusion gene into mutant eggs, as this construct has been shown to stimulate growth of normal mice¹³. Forty-one animals developed from microinjected eggs; seven *lit/lit* mice carried the foreign gene (Table 2).

By 5 weeks of age, six of the seven transgenic *lit/lit* mice were already twice mutant size, and by 16 weeks were approximately 1.5 times the size of *lit/+* mice (Table 2). Thus *MT-rGH*

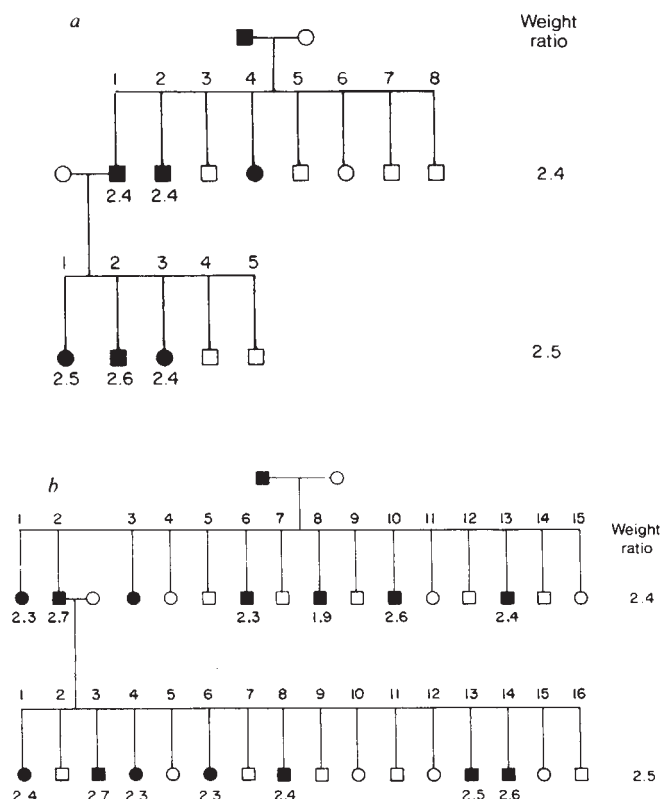


Fig. 1 Pedigree from: a, 41-1; b, 54-1 males. Mice that carry the *MT-rGH* genes are indicated by the solid symbols. Squares represent males and circles females. Numbers above symbols designate animal number and numbers below solid symbols the weight ratio of a *MT-rGH* mouse to control littermates at 6 weeks of age. Female 3 in the *F*₁ generation of 54-1 and female 4 in the *F*₁ generation of 41-1 died before body weights were taken.

Table 1 Transfer of *GH* structural genes to mice

Injected genes	DNA length (kb)	No. of transgenic mice	No. of gene copies	Rat or human GH in serum	Body weight	
					♂	♀
<i>rGH</i>	7.6	8	1–250	ND	27.4 ± 2.1	22.3 ± 1.8
<i>hGH_N</i>	2.6	8	1–10	ND	27.1 ± 2.3	22.7 ± 1.1
<i>hGH_N</i> , <i>hPL_L</i> , <i>hPL_A</i>	43.0	16	1–50	ND	27.6 ± 1.2	21.5 ± 1.5
Control	—	—	—	—	27.5 ± 3.2	22.2 ± 1.7

A 7.6-kilobase (kb) *Bam*HI linearized fragment containing the entire *rGH* structural gene^{8,19} was isolated and about 1,000 copies were microinjected into the male pronucleus of eggs from C57 × SJL hybrid crosses²⁰. The 2.6-kb *Eco*RI linearized fragment containing the entire *hGH* structural gene (*hGH_N*)^{9,21} or a cosmid fragment containing three genes of the *hGH* gene family (*hGH_N* and two human placental lactogen genes, *hPL_L* and *hPL_A*; *cGH4*, 43 kb)²¹ were isolated and about 1,000 copies microinjected. The number of foreign structural genes per cell was estimated by DNA dot hybridization with a nick-translated 1.0-kb *Pst*I probe for *rGH* and a 1.0-kb *Pvu*II probe for *hGH_N* and *cGH4*. The radioactivity was then measured by scintillation counting^{11,13}. Serum rat GH or hGH were determined by radioimmunoassay of duplicate samples using either rat¹⁹ or human¹¹ GH standards respectively. The GH antiserum used interacts with both rat and mouse GH; therefore 'ND' indicates no significant difference between the serum GH concentration of *rGH* transgenic (mean ± s.d. 254 ± 54) and control (319 ± 50) mice. The concentration of hGH in *hGH_N* and *cGH4* mice was lower than the limit of detection (~10 ng ml⁻¹) and is listed as 'ND'. Body weight is given as mean ± s.d. at 11 weeks of age; *n* = 42 animals.

Table 2 Expression of *MT-rGH* fusion gene in transgenic *little* mice

Animal	Sex	Genotype	Gene copy (No. per cell)	Serum GH (ng ml ⁻¹)	Body weight (g)	Ratio
20-1	♀	<i>lit/lit</i> ¹	4	2,600	50.0	4.0
41-1	♂	<i>lit/lit</i> ²	17	31,200	41.8	2.9
41-2	♀	<i>lit/lit</i> ²	98	29,300	38.0	3.0
41-3	♀	<i>lit/lit</i> ²	1	1,463	12.9	1.0
54-1	♂	<i>lit/lit</i> ¹	8	2,764	43.7	3.0
65-1	♀	<i>lit/lit</i> ¹	10	1,630	39.0	3.1
65-2	♀	<i>lit/lit</i> ¹	12	6,540	37.5	3.0
Control	♂	<i>lit/lit</i>	—	66 ± 11	14.6 ± 1.8	
Control	♀	<i>lit/lit</i>	—	76 ± 8	12.6 ± 0.4	
Control	♂	<i>lit/+</i>	—	254 ± 90	28.8 ± 2.3	
Control	♀	<i>lit/+</i>	—	254 ± 60	23.0 ± 1.9	
Control	♂	<i>+/+</i>	—	110 ± 18	31.0 ± 0.7	
Control	♀	<i>+/+</i>	—	240 ± 55	25.9 ± 1.0	

A 5.0-kb linear fragment of the fusion gene *MT-rGH*¹³ containing the mouse *MT-I* regulator/promoter region fused to *rGH* gene was injected into fertilized one-cell eggs collected from superovulated²² *lit/lit* females crossed with either *lit/lit* males or hypodactyl males carrying the little gene (*Hd +/+lit*). The male pronuclei of 690 fertilized eggs were microinjected with approximately 2 pl of buffer containing 300–600 copies of DNA, and 494 eggs were transferred into the oviducts of pseudopregnant recipients²⁰. The animals were weaned and total nucleic acids extracted from a piece of tail and used for dot hybridization with a probe corresponding to exons 1 and 2 of *rGH* gene. Digestion of the DNA by *Sst*I or *Stu*I and Southern blotting confirmed the presence of *MT-rGH* genes and indicated that, in all animals with multiple copies, the foreign genes were integrated as tandem arrays (data not shown)²⁰. For the genotype, mice that developed from eggs obtained from the cross mutant *lit/lit* females × *lit/lit* males are indicated by superscript 1 and those developing from crosses between *lit/lit* females and *Hd +/+lit* males (hypodactyl males carrying the little gene) are indicated by superscript 2. Mutant males have delayed sexual maturation and show a marked degree of infertility¹. This suboptimal fertility was improved by implanting pituitaries from heterozygous mice into mutant males, thereby allowing us to make homozygous crosses for egg collection. The hypodactyl gene which is closely linked to little gene locus¹ was introduced into the little colony by crossing *lit/lit* females with *Hd +/+lit* males and using the resulting hypodactyl male offspring (*Hd +/+lit*) for stud with *lit/lit* females. Mice resulting from this cross are either *+lit/+lit* (five-toed and dwarf) or *Hd +/+lit* (hypodactyl and normal). The number of *MT-rGH* genes per cell was estimated by DNA dot hybridization with a nick-translated *Sst*I–*Bgl*II probe corresponding to the mouse *MT-I* promoter/regulator, and scintillation counting²³. Serum GH levels were determined by radioimmunoassay of duplicate samples using GH standards¹⁹. The antibody used for *rGH* determinations binds both rat and mouse GH; therefore appropriate control values should be subtracted from transgenic GH values. Body weights were compared at 16 weeks of age. The weight ratio compares transgenic mutant body weight to control mutant mice of the same sex. Values for controls are mean ± s.e.m., with *n* ≥ 8 in each group.

expression in transgenic mutants not only restores growth but changes their phenotype from dwarfs to giants. The absence of a correlation between copy number or gene dosage and growth rate has also been observed in other *MT-rGH* mice (unpublished) and *MT-hGH* mice¹¹ and may result, at least in part, from only one or a few genes in the tandem arrays actually being expressed.

Serum GH levels in *MT-rGH* transgenic and control mice were quantified to see whether the accelerated growth correlated with elevated serum GH (Table 2). Mutant mice had approximately one-third the quantity of GH found in *lit/+* mice. All seven of the transgenic mutant mice had elevated serum GH ranging between 20 and 400 times greater than *lit/lit* control mice and 5 and 120 times greater than *lit/+* and *+/+* controls. The one transgenic mouse which failed to grow also had elevated serum GH. Transgenic mice with this characteristic have occurred previously in both *MT-rGH* (unpublished) and *MT-hGH*¹¹ experiments, indicating that although an immunoreactive product is produced, its biological potency is not assured.

To determine whether *MT-rGH* was stably integrated into the host chromosome making the phenotype heritable, two transgenic *lit/lit* males, 41-1 and 54-1 (Table 2), were bred with *lit/lit* females (Fig. 1). Both males transmitted their characteristic number of copies of fusion gene (data not shown) to approximately half of their offspring, indicating that multiple copies were integrated into a single chromosomal location. In both lines, the fusion gene has been transmitted into the second generation as well; every animal carrying *MT-rGH* genes was more than twice mutant size by 6 weeks of age.

To establish when transgenic mutants develop a sensitivity to elevated serum GH, we bred 54-1-2 ♂ with *lit/lit* females (Fig. 1) and compared the growth rates of transgenic and normal mutant offspring and *+/+* controls (Fig. 2). At about 40 days of age the growth of mutant mice essentially ceases. In contrast, transgenic mutant mice enter an accelerated growth phase at about 20 days of age and by 90 days they are approximately three times the size of nontransgenic littermates. Transgenic mutants surpass normal body weight by 30 days of age and by 90 days are more than 1.5 times normal weight.

All the transgenic males in the *F*₁ generation of 54-1 have been bred and have sired at least two litters. Thus in contrast to mutant males where only 50% sire one litter, 30% sire second litters and third litters are rare; the fertility of transgenic mutant males is improved by *MT-rGH* expression. Transgenic mutant females (with the exception of 41-3, Table 2) were also bred to *+/+* males and all four were infertile. Although control *lit/lit* females show delayed sexual maturation, they eventually reach full fertility¹. We also assessed the effects of extra-pituitary GH production on the fertility of *MT-GH* transgenic C57 × SJL hybrid mice. *F*₀ males exhibited slightly reduced fertility (15 out of 20 were fertile). The fertility of *F*₁ *MT-GH* male mice was

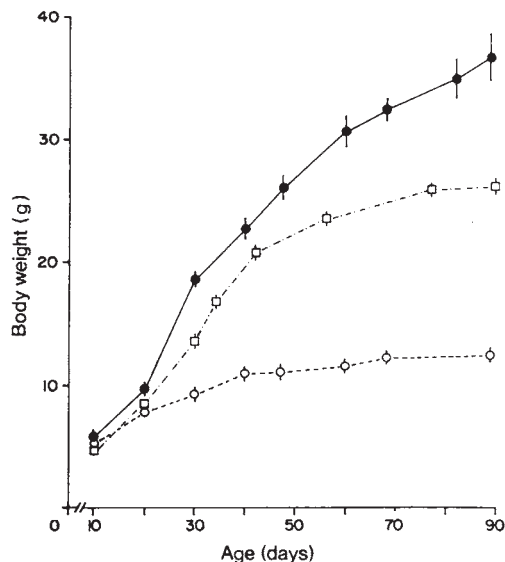


Fig. 2 Growth curves of MT-rGH *lit/lit* mice, nontransgenic littermates and control C57Bl/6J mice. One litter (eight pups) from the F₂ generation of 54-1 (see Fig. 1) was marked 1 week after birth and their weights were recorded periodically. Hybridization of tail DNA with a 0.38-kb *Xho*I-*Pvu*II rGH probe revealed five offspring inherited the MT-rGH gene (●) and three did not (○). □, Weights of C57Bl/6J mice. Mean body weights ± s.e.m. are indicated at each point.

normal with 11 out of 12 siring multiple litters. In contrast, the fecundity of F₀ transgenic females was dramatically reduced: only 1 of 10 of the MT-GH females bred was fertile. Thus whereas excess GH in transgenic mutant males improves fertility and has a negligible effect on the fertility of other MT-GH males, it dramatically reduces the fertility of both transgenic mutant and +/+ females. The mechanisms by which GH influences fertility are unexplored.

Several different *Drosophila* null mutants have been corrected recently by introducing wild-type gene counterparts into the germ line by their injection as part of P-element vectors into mutant eggs¹⁴⁻¹⁶. In the case described here, the primary defect is unknown. The *lit* mutation probably affects the regulation of expression of GH gene rather than gene itself^{2,3}. Therefore, we attempted to correct the phenotypic defect in GH production by supplying a GH gene attached to an alternate promoter so that it would be independent of normal GH regulation. Although this approach has remedied the diminutive size of *lit/lit* mice as well as improved the fertility of males, we have uncovered an unsuspected deleterious effect of chronic elevated GH production on female fertility.

Although it seems feasible to correct certain genetic defects in animals, the application of the present technology to humans is inappropriate for several practical reasons. First, the techniques are inefficient; only about 1% of the injected eggs develop into mice that express the gene. Second, because the integration site of the foreign DNA is unpredictable, it is not possible to replace mutant DNA sequences with normal sequences. Thus the mutant gene will not be eliminated from the gene pool and will probably segregate independently of the foreign gene in the next generation. Furthermore, the unpredictability of integration means that it is possible that the foreign DNA could disrupt a normal gene, thereby creating a new mutation^{10,17,18}. Current estimates are that about 20% of transgenic mice harbour recessive mutations^{10,17}. These considerations, coupled with the fact that for most genetic diseases only one of four offspring are at risk, argue against application to humans.

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Live recombinant vaccinia virus protects chimpanzees against hepatitis B

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Hepatitis B virus (HBV) is an important human pathogen responsible for over 200 million cases of chronic infection, many of which progress to hepatocellular carcinoma. Although HBV cannot be propagated in tissue culture, highly effective subunit vaccines obtained from the plasma of chronically infected patients have been developed and licensed¹⁻³. Such vaccines are safe but their expense and limited quantities make them unavailable to most Third World countries. Other approaches to vaccine construction, including purification of the HBV surface antigen (HBsAg) from genetically engineered eukaryotic cells⁴⁻¹³ and the synthesis of peptides predicted from the nucleotide sequence of the HBsAg gene¹⁴⁻¹⁹, are still under evaluation. Another potential application of recombinant DNA technology to vaccine development is the use of live virus vectors to express foreign genes²⁰⁻²⁵. An infectious vaccinia virus recombinant that expressed the HBsAg in animal cells and which stimulated the production of antibody to HBsAg (anti-HBs) in rabbits represented a novel candidate vaccine of this class^{22,23}. As a continuation of our earlier study, we now present evidence that chimpanzees vaccinated with a live recombinant vaccinia virus were protected against hepatitis following challenge with HBV.

The construction of vHBs4, the recombinant vaccinia virus used for these experiments, has been described elsewhere²². Briefly, a 1,350-base pair DNA fragment of HBV strain *adw*, containing the entire gene coding for HBsAg, was fused to an early vaccinia virus promoter and inserted into the coding sequence of the vaccinia virus thymidine kinase (*tk*) gene. The chimaeric gene was inserted into vaccinia virus by homologous recombination *in vivo* and infectious TK⁻ recombinant virus was isolated.

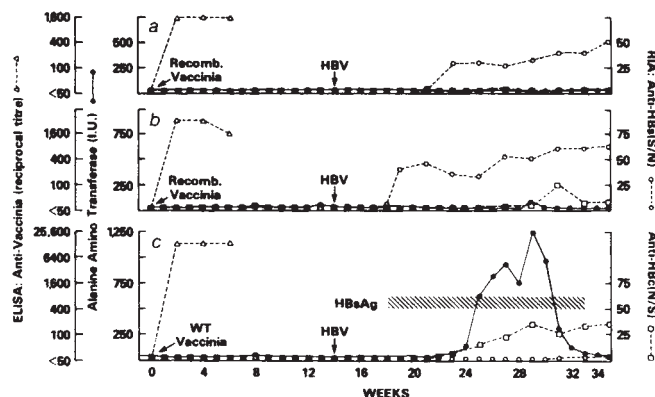


Fig. 1 Response of chimpanzees to intradermal vaccination with live recombinant virus vHBs4 or wild-type vaccinia virus, followed by challenge with live HBV. Chimpanzee 66 (a) and chimpanzee 67 (b) received recombinant vaccinia virus; chimpanzee A-98 (c) received wild-type vaccinia virus. Alanine aminotransferase activity is expressed as reciprocal dilutions; anti-HBs and anti-HBc are expressed as the ratio of sample c.p.m. to negative control c.p.m. (S/N) and negative control c.p.m. to sample c.p.m. (N/S) respectively. Positive HBsAg values (>2.1 P/N) are shown as cross-hatching; between weeks 19 and 32, chimpanzee A-98 had P/N values ranging from 56 to 155.

Three chimpanzees (*Pan troglodytes*) weighing 14–25 kg were housed and maintained as described previously²⁶. None showed serological evidence of prior exposure to HBV, as determined with commercial radioimmunoassays for HBV markers (HBsAg, AUSRIA; anti-HBs, AUSAB; anti-HB core antigen, anti-HBc, CORAB; anti-HBc IgM, CORZYME-M, Abbott Laboratories). Also, the animals had no other evidence of hepatitis, as judged by normal levels of alanine aminotransferase, aspartate aminotransferase and isocitric dehydrogenase enzymes.

To test the ability of vaccinia virus recombinant vHBs4 to protect chimpanzees against hepatitis, 10^8 plaque forming units (PFU) in 0.1 ml were administered intradermally in the upper back of two animals (66 and 67). A third chimpanzee (A-98) was vaccinated with 10^8 PFU of wild-type vaccinia virus. All three chimpanzees developed primary vaccinia lesions at the sites of vaccination. The two chimpanzees vaccinated with the recombinant virus had dermal lesions of diameters 24 and 22 mm, respectively. The chimpanzee vaccinated with the wild-type vaccinia developed a larger primary lesion of 37 mm diameter. A secondary vaccinia lesion developed in the right axilla of this animal. The greater severity of infection in the chimpanzee receiving wild-type virus, compared with that of chimpanzees receiving recombinant virus, was also reflected in a four- to eight-fold higher antibody titre to vaccinia virus when tested by enzyme-linked immunosorbent assay (ELISA)²⁷ (Fig. 1). Correlation of the TK⁻ phenotype of recombinant vaccinia virus with attenuated pathogenicity will be presented elsewhere.

Following vaccination, weekly serum samples were obtained from the three chimpanzees and tested for biochemical evidence of liver disease and serological evidence of exposure to hepatitis B viral antigens as described above. All sera were negative for elevations of liver enzyme activity and for evidence of exposure to HBV antigen except for one weak positive but repeatable 2.5 P/N value (ratio of positive c.p.m. to negative control c.p.m.) for anti-HBs 8 weeks after vaccination of chimpanzee 67 (Fig. 1).

Fourteen weeks after vaccination, all three animals were challenged intravenously with $10^{3.5}$ chimpanzee ID₅₀ units of live HBV (subtype ayw, strain MS-2), a dose and strain of HBV which has consistently produced hepatitis B in chimpanzees^{26,28}. Figure 1 shows that chimpanzee A-98 (vaccinated with wild-type vaccinia) developed typical hepatitis B. Circulating HBsAg appeared 4 weeks after HBV challenge and reached a peak P/N value of 155. Anti-HBc and biochemical evidence of hepatitis occurred after 8 and 9 weeks, respectively. HBsAg levels declined

and disappeared by 19 weeks after challenge, coincident with the appearance of anti-HBs.

In contrast, chimpanzees 66 and 67 (vaccinated with recombinant virus) had no detectable HBsAg or biochemical evidence of hepatitis. Instead, anti-HBs appeared at 4–7 weeks after challenge and rapidly rose to high levels that persisted throughout the experiment. Both chimpanzees developed low levels (3.8–8.8 N/P) of anti-HBc, 21 or 27 weeks post-challenge, indicating that they had experienced inapparent infections with HBV. As a follow-up, the livers of the two chimpanzees inoculated with recombinant vaccinia virus were biopsied at 11 months after HBV challenge. There was no evidence of acute or chronic hepatitis.

Sera from chimpanzees 66 and 67 were analysed for the immunoglobulin class of anti-HBs by sucrose gradient centrifugation followed by radioimmunoassay of the fractions containing IgM or IgG immunoglobulins for anti-HBs activity. Two weeks after detection of anti-HBs in chimpanzees 66 and 67, IgM and IgG anti-HBs activities were present in approximately equal proportions (data not shown). Late convalescent sera (14–17 weeks after seroconversion) contained only IgG anti-HBs. Thus, the immune reaction had features characteristic of both primary and anamnestic responses. Sera obtained from chimpanzees 66 and 67 during the early phase of the immune response (2–6 weeks after seroconversion) were tested by radioimmunoassay²⁹ for the subtype specificity of the anti-HBs: only anti-a was detected.

This study demonstrated that chimpanzees receiving a single intradermal vaccination with a live recombinant vaccinia virus that expresses HBsAg were protected against hepatitis B. Following a challenge with HBV of a heterologous subtype, the vaccinated animals experienced a mild inapparent infection characterized only by seroconversion to anti-HBs and anti-HBc. The marginal primary antibody response of chimpanzees 66 and 67, when compared with the anti-HBs response of rabbits following vaccination with this same recombinant vaccinia virus, is in keeping with the relatively poor response of primates to a single dose of HBsAg in the absence of adjuvants³⁰. Although the chimpanzees had little or no circulating anti-HBs after vaccination, they were immunologically 'primed'. Consequently, they showed a brisk and sustained antibody response, presumably due to newly synthesized HBsAg, following challenge with live HBV. The early detection of IgG anti-HBs and the anti-a subtype specificity are consistent with the anamnestic nature of the response. Priming of neutralizing antibody production by vaccination of rabbits with synthetic peptides of poliovirus structural protein VP1 has been reported³¹, but has not been demonstrated previously for HBV.

In conclusion, these studies indicate the feasibility of using a recombinant vaccinia virus as a live hepatitis B vaccine. Current research is directed towards increasing HBsAg expression by using stronger vaccinia virus promoters.

Since submission of this manuscript, additional vaccinia virus recombinants that express prokaryotic chloramphenicol acetyltransferase³², herpesvirus glycoprotein D (ref. 33), HBsAg³³ and malaria sporozoite antigen³⁴ have been described.

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Defect in the generation of light-chain diversity in bursectomized chickens

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The avian bursa of Fabricius has been regarded as a central organ for B-cell development^{1,2}, but there is controversy about the existence of other sites for differentiation of B cells^{3–6}. We have recently shown that chickens surgically bursectomized as early embryos, before the bursal primordium appears, can produce cytoplasmic, surface and serum immunoglobulins of IgM, IgG and IgA classes but are unable to generate specific antibodies in response to antigen^{7–9}. We have therefore examined the structure and diversity of immunoglobulins of bursectomized chickens. Analysis of serum IgG revealed normal γ -heavy chains but altered light chains with more basic and less diverse isoelectric points than normal. These light chains may represent germ-line specificities not diversified by somatic mutations. Thus the bursa of Fabricius appears not to be necessary for the production of immunoglobulin molecules as such but to function in the creation and expansion of the antibody repertoire, possibly by providing a microenvironment for somatic mutations.

Chickens were surgically bursectomized after 60 h incubation by cutting off the tail caudally to the leg buds. Bursectomy was ascertained by careful microscopic examination at the autopsy at 10 weeks of age^{7,8}. All bursectomized chickens had detectable amounts of serum immunoglobulins; mean concentrations of serum IgM and IgA were normal, whereas that of serum IgG was 1/10 of the control value⁹. In spite of immunoglobulin production, the bursectomized chickens did not respond to repeated immunizations with levan, *Escherichia coli* lipopolysaccharide, tetanus toxoid, dinitrophenyl-bovine serum albumin, brucella, diphtheria, pertussis, salmonella, sheep red blood cells and human γ -globulin^{7,9}. Also all the tests made to detect natural antibodies to major histocompatibility complex (MHC) products, rabbit red blood cells, faecal bacteria and phosphorylcholine, and autoantibodies to the kidney, thyroids and liver, were totally negative. Antibodies to bursal structures were not observed⁹. The total lack of inducible or spontaneous antigen-specific

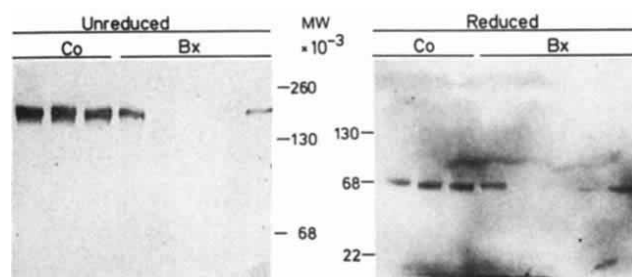


Fig. 1 Molecular weight of serum IgG and γ -chain of the control (Co) and bursectomized (Bx) chickens. Serum samples (1 μ l) were examined in non-reducing (left) or reducing (right) conditions by SDS-PAGE¹⁰ by using a 5% homogeneous or 5–22.5% gradient gel, respectively. After electrophoresis, proteins were transferred to a nitrocellulose sheet¹² (HAWP 000 10; Millipore). The sheet was saturated for 1 h with 20% fetal calf serum (FCS) diluted in Tris-buffered 0.9% NaCl (TBS) and then incubated for 2 h with rabbit antiserum against chicken γ -chain⁸ diluted in 20% FCS-TBS. After extensive washings with 0.1% Triton-X-100-TBS for 1 h, alkaline phosphatase conjugated pig anti-rabbit IgG (Orion Diagnostica) was added and incubation was continued overnight. After washings with 0.1% Triton-X-100-TBS phosphatase activity on the sheet was visualized with α -naphthyl phosphate as a substrate and 4-amino-diphenylamine diazonium sulphate as a staining dye¹¹. Pepsin-digested collagen β - and α -chains of type 1 of molecular weight (MW) 260,000 (260K) and 135K, bovine serum albumin (68K) and trypsin inhibitor (22K) were used as molecular weight standards.

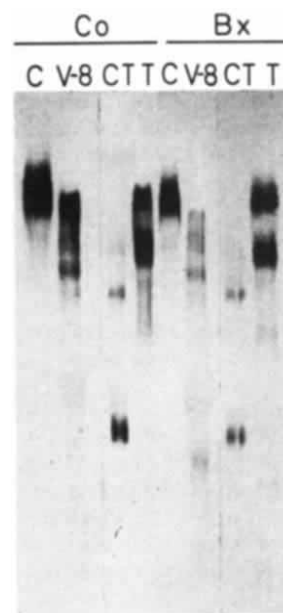


Fig. 2 γ -Chain fragments of one control (Co) and one bursectomized (Bx) bird after enzymatic digestion with *Staphylococcus aureus* V-8 protease (V-8), chymotrypsin (CH) or trypsin (T); control incubation without any enzyme (C). Sodium sulphate precipitated serum immunoglobulins in SDS-buffer were subjected to enzymatic digestion for 2 h at 37 °C according to Cleveland *et al.*²¹ All enzymes were used in concentrations of 25 μ g ml⁻¹. After digestion proteins were fractionated by using a 15% homogeneous SDS-PAGE. Transfer to a nitrocellulose sheet and identification of γ -chain fragments were performed as described in Fig. 1 legend.

antibody responses, given near normal immunoglobulin levels, suggested that the immunoglobulins produced by bursectomized chickens might be abnormal in structure. To examine this possibility, we compared serum IgG of the bursectomized and control chickens at the age of 10 weeks. Serum proteins were electrophoresed in SDS-polyacrylamide gel electrophoresis (PAGE)¹⁰ or electrofocused in thin layer agarose gel¹¹, and then

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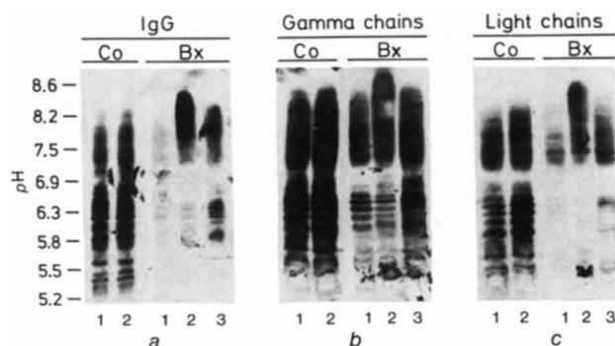


Fig. 3 *a*, Diversity of IgG; *b*, γ -chains; *c*, light chains of the control (Co) and bursectomized (Bx) chickens. Only bursectomized birds with serum IgG >10% of the control value were selected for diversity analysis. Serum samples (2 μ l) were electrofocused in 1% agarose gel in the absence (*a*) or presence (*b*, *c*) of 0.1 M mercaptoethanol¹¹. After electrofocusing, proteins were transferred to a nitrocellulose sheet¹¹ and then the sheet was stained with the following antibodies. *a*, *b* Stained with the same procedure as in Figs 1 and 2 using rabbit antiserum against chicken γ -chain⁸ at the first step. *c*, First incubated with monoclonal mouse anti-chicken light chain¹³, then with rabbit anti-mouse IgG (Dako-immunoglobulins) and finally with alkaline phosphatase-conjugated pig anti-rabbit IgG (Orion Diagnostica). The pH gradient of the gel as measured with a surface electrode is indicated on the left. Light chains of both normal chickens reveal 26 separate bands at pH values 5.2–6.9 (concentration of total serum immunoglobulins 20.4 and 12.3 mg ml⁻¹) whereas two of the bursectomized chickens show 11 bands (concentration of total serum immunoglobulins 6.8 and 2.3 mg ml⁻¹) and the third showed 20 bands (concentration of total serum immunoglobulins 14.3 mg ml⁻¹) in this same area, indicating limited diversity of bursectomized birds show the same or even greater light-chain diversity than the controls. The mean isoelectric points of the light chains of bursectomized chickens are more basic than normal. In contrast, γ -chains of the bursectomized chickens have normal diversity.

transferred to nitrocellulose sheets^{11,12}. Immunoglobulins on the sheets were identified by an indirect immunoenzymatic staining¹¹ using polyclonal rabbit antibodies to γ -chain⁸ and monoclonal mouse antibodies to light chain¹³ (gift from Dr Max Cooper).

Bursectomized chickens had entire IgG molecules containing both heavy and light chains of apparently normal molecular weight (for γ -chain ~70,000 and for whole molecule ~190,000; Fig. 1). γ -chains of the bursectomized and control chickens showed identical cleavage patterns with three different proteases (Fig. 2), which suggests that their major structural fragments are similar and that they are expressed after similar gene rearrangement. Thus, IgG from bursectomized chickens exhibited no detectable structural abnormalities. However, whole IgG molecules of the bursectomized chickens had more basic isoelectric points than those of the controls (Fig. 3*a*). This was not due to a change in γ -chains, because they showed a normal diversity in isoelectric points (Fig. 3*b*). Instead, bursectomy decreased the amount of acidic light chains to an almost undetectable level without having any corresponding effect on the amount of basic light chains (Fig. 3*c*). Thus, alterations in the isoelectric spectra of the whole bursectomized IgG result from alterations in the light chains. This finding is clarified further in Fig. 4. The results clearly suggest abnormally limited diversity in these light chains.

Normal concentration of serum immunoglobulins does not necessarily imply normal immunoglobulin diversity and antigen binding capacity¹⁴. In this respect, the bursectomized chickens closely resemble human patients suffering from antibody deficiency with normal serum immunoglobulins^{15,16}. Our finding of limited diversity of light chains in the bursectomized chickens is supported by the previous findings of Huang and Dreyer¹⁷,

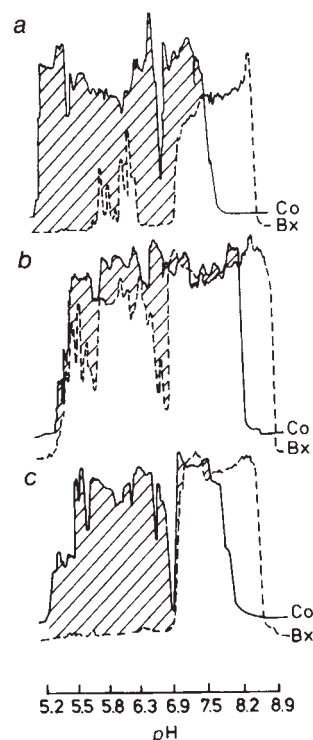


Fig. 4 Densitograms of the isoelectric focusing patterns of: *a*, whole IgG; *b*, gamma chains; *c*, light chains of one control (Co) and one bursectomized (Bx) chicken. These densitograms are made from isoelectric focusing patterns of the chickens Co 1 and Bx 2 in Fig. 3.

who used testosterone bursectomy *in ovo* at day 11 of incubation. They concluded that bursectomy at day 11 of incubation blocks the generation of new clones of B cells. However, when bursectomy is performed 4 days after hatching a normal diversity of antibodies to human serum albumin develops¹⁸.

Both somatic recombination and somatic mutation diversify the genetic information coded by the germ line genome. Somatic mutation, which alters bases throughout the sequences coding for the variable region, amplifies the diversity arising through recombination¹⁹. The isoelectric spectra of light chains of bursectomized chickens indicate that they consist of more basic amino acids than normal and may represent germ-line specificities. The antigen specificities of these antibodies have not yet been determined. The number of genes coding light-chain variable regions is not known in the chicken, but it is very probable that the number is small as in the human, and the diversity is generated mostly by somatic mutations²⁰. Abnormality only in the light chains of the bursectomized chickens, on the other hand, indicates either that the specific influence of the bursa of Fabricius on B-cell development occurs late in the history of the B-cell clones, or that the normal diversity of the γ -chains observed in isoelectric focusing is generated with high numbers of variable-region genes.

Thus, our findings in bursectomized chickens of immunoglobulins without detectable antigen specificities and of abnormal diversity of light chains suggests that the bursa of Fabricius creates and expands the antibody repertoire by regulating somatic diversification, especially somatic mutations.

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Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins

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J chain is a polypeptide of molecular weight (M_r) ~15,000 common to human dimeric IgA and pentameric IgM^{1,2}. These immunoglobulin polymers show a high affinity for secretory component (SC) *in vitro*^{3,4}, a feature that, in some studies, has been claimed to be a function of the J chain^{5,6}. SC is a glycoprotein of M_r ~80,000 which is expressed on the basolateral surfaces of secretory epithelial cells^{7,8} where, according to a current hypothesis, it may act as a receptor for dimeric IgA and pentameric IgM⁹ which are selectively transported through secretory epithelial cells into exocrine fluids^{10,11}. Previous studies, however, have not excluded the possibility that secretory cells express isotype-specific Fc receptors for IgA and IgM which may be involved in epithelial transport. We now report that the adsorption of immunoglobulin polymers to SC-expressing epithelial cells depends solely on a J chain-determined binding site. This finding lends biological significance to the striking J-chain expression shown by immunoglobulin-producing immunocytes in secretory tissues^{12–14}.

Almost 20 years ago Tomasi *et al.*¹⁵ showed that the major immunoglobulin class present in human secretions is dimeric IgA covalently bound to an epithelial component now called SC. Pentameric IgM is similarly enriched in most exocrine fluids in contrast to IgG¹⁰; and secretory IgM is also associated with SC, although not in a covalently stabilized complex¹¹.

At least seven models have been proposed for the epithelial transport of IgA (reviewed in ref. 9). The common transport model for dimeric IgA and pentameric IgM proposed by one of us in the early 1970s^{16,17} has subsequently gained considerable support from observations of both normal⁸ and neoplastic^{18,19} living epithelial cells. This model postulates that the J chain and SC represent the 'lock and key' in the selective external transport. It has recently been shown that SC is synthesized as a transmembrane protein of M_r ~95,000 (ref. 20). This larger SC precursor may alone, or in combination with other binding factors, constitute the actual receptor.

As it is known that human hepatocytes contain an IgA-binding site unrelated to SC⁹ and that subsets of T and B lymphocytes^{21,22}, monocytes²³, alveolar macrophages²⁴ and

granulocytes²³ express Fc α receptors, we believed it important to examine whether this is the case also for human secretory epithelial cells. In addition, we wanted to test directly the role of J chain in immunoglobulin binding to cell membrane-associated SC.

Experiments were performed with cells from the colonic carcinoma line designated H29 (ref. 25). The subclone HT29.E10 (used with the permission of Dr K. E. Mostov; ref. 20) was maintained as monolayers in 75 cm² Costar flasks at 37 °C with an atmosphere of 5% CO₂ in McCoy's Modified 5A medium supplemented with 15% fetal bovine serum, 2 mM glutamine, 50 units ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin. The cultures were split by 7-min treatment with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA (Trypsin-EDTA; Flow), washed and seeded into Leighton tubes. Coverslips from 2–6-day-old cultures were divided into small areas that were subjected to tests with various human protein preparations (Table 1).

Direct immunofluorescence showed that only 20–40% of the living cells expressed substantial amounts of SC. After preincubation with polymeric IgA containing appropriate amounts of J chain (>4 mg per 100 mg), paired staining showed strict correspondence between SC expression and IgA binding (Fig. 1a); a positive result was obtained with staining end points of <0.02 g l⁻¹ for unlabelled and <0.01 g l⁻¹ for fluorescein-labelled preparations (Fig. 2). The only exception was colostral IgA which did not produce binding below 1 g l⁻¹ (Fig. 1c). Monomers of IgA having no J chain showed no adsorption to the cells (Fig. 2b), even when tested at ≥3.5 g l⁻¹ (Fig. 2). Conversely, three monomer preparations with small amounts of J chain showed binding between 0.3 and 1.8 g l⁻¹ (Fig. 2), probably reflecting the presence of contaminating polymers. Four IgA polymers deficient in J chain produced staining end points comparable to the latter three monomer preparations when J-chain content and percentage SC-binding capacity were taken into account (Figs 1d, 2). The same was true for a J chain-deficient polymeric IgM in contrast to the three pentamers with normal J-chain content (Figs 1e, f, 2). On the whole, there was good agreement between the SC-binding capacity of the various preparations and their staining end points when tested on living cells (Fig. 2).

No binding of IgA or IgM was observed with epithelial cells lacking SC (Fig. 1a, e). Conversely, IgG at 4 g l⁻¹ was largely adsorbed to such cells (Fig. 1g), probably reflecting the presence of Fc γ receptors, which may be expressed by carcinoma cells²⁶. This finding indicated that the amount of these receptors might be related to cellular de-differentiation resulting from clonal diversification as preservation of SC expression is found mainly in well-differentiated colonic carcinomas²⁷.

In previous binding studies with the HT29 cell line, no dose-response analyses were performed. One study included dimeric IgA, monomeric IgA and IgG only at 0.2 g l⁻¹ (ref. 19) and another used a much higher (4 g l⁻¹) concentration¹⁸. Although no binding was reported for monomeric IgA, the possibility could not be excluded that the positive results obtained with polymers were due to isotype receptors because paired staining for ligand and SC was not performed. Indeed, it has been shown that the Fc α receptors present on granulocytes show much higher (~10 times) binding activity for dimeric than for monomeric IgA²³. We do not believe that tests with colostral IgA are an appropriate control; its incorporated SC could, by steric hindrance, have interfered with binding to putative Fc α receptors. Blocking by preincubation with antibodies to SC¹⁸ is, using a similar reasoning, an unsatisfactory control.

Thus, for the first time we have excluded the possibility that 'conventional' Fc α and Fc μ receptors are significantly involved in the adsorption of immunoglobulin polymers to human secretory epithelial cells; a J chain-determined binding site specific for SC seems to be the decisive structure. Although much work has been done to elucidate a role for the J chain in B-cell differentiation, there is as yet no clue to this end²⁸. Immunoglobulin-producing cells may express cytoplasmic J chain regardless of concurrent isotype¹²; but the combination

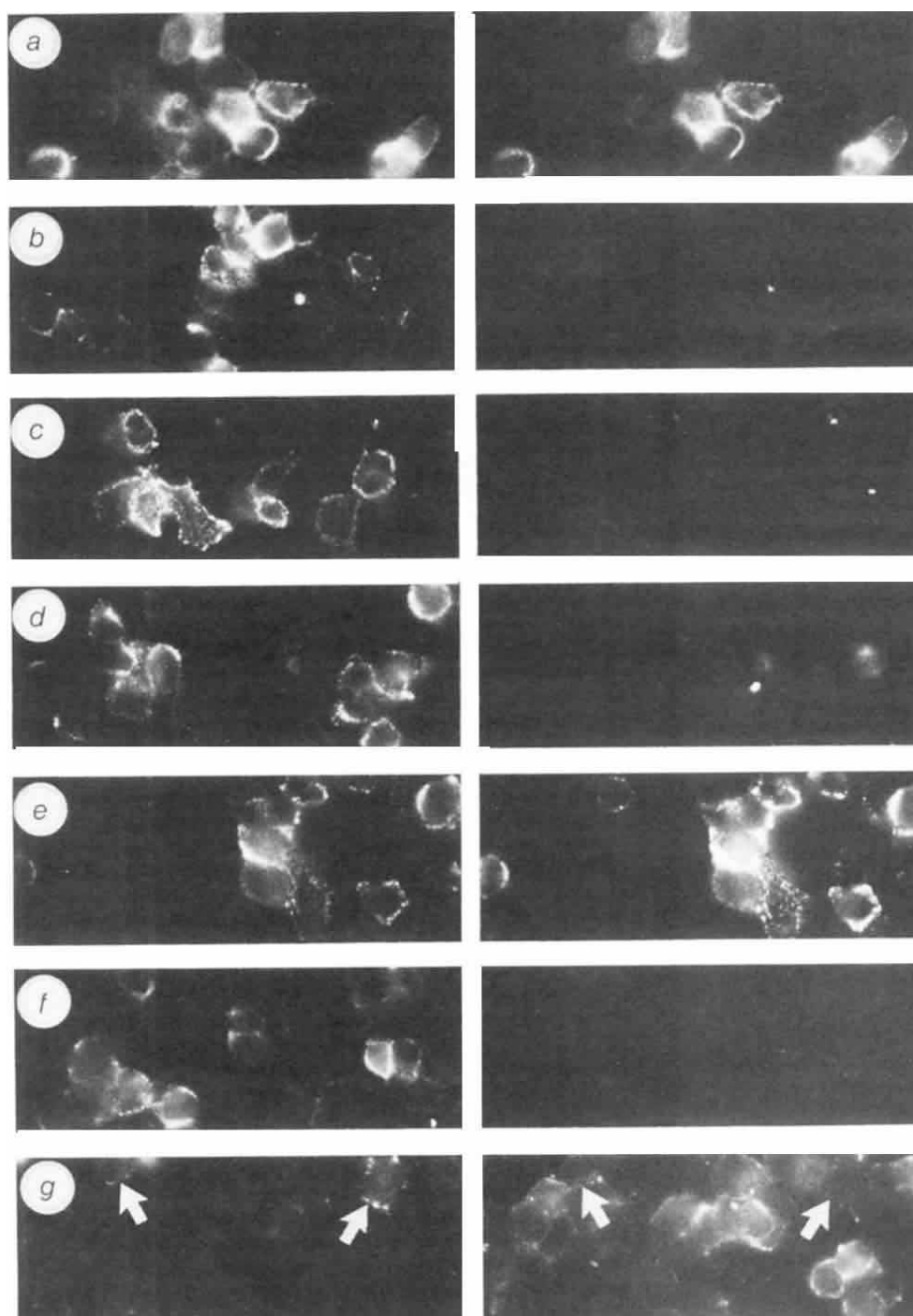


Fig. 1 Paired immunofluorescence staining of vital HT29.E10 monolayer preparations to demonstrate concurrent surface expression of SC (left panel, 10-s exposure of rhodamine emission) and binding of IgA, IgM or IgG (right panel, 20-s exposure of fluorescein emission). *a*, Binding of fluorescein-labelled polymeric IgA at 0.15 g l^{-1} . *b*, Lack of binding of fluorescein-labelled monomeric IgA at 1.2 g l^{-1} . *c*, Absence of binding of fluorescein-labelled colostral IgA at 1.0 g l^{-1} . *d*, Lack of binding of J chain-deficient polymeric IgA at 2.9 g l^{-1} . *e*, Binding of polymeric IgM at 0.25 g l^{-1} . *f*, Absence of binding of J chain-deficient polymeric IgM at 0.25 g l^{-1} . *g*, Binding of IgG at 4.0 g l^{-1} obtained mainly with SC-negative cells; the locations of two SC-positive cells are indicated by arrows. $\times 390$.

Methods: Protein binding to living cells was tested by incubating unfixed monolayers at room temperature for 45 min with various concentrations of human immunoglobulin preparations which were applied either unlabelled or conjugated with fluorescein isothiocyanate as indicated. Dilutions (4.0 – 0.005 g l^{-1}) were done in phosphate-buffered isotonic saline (PBS), pH 7.5, containing bovine serum albumin (120 g l^{-1}). After washing in PBS (10 min), the monolayers were incubated (30 min) with a combination of rhodamine conjugate specific for SC and fluorescein conjugate specific for the appropriate immunoglobulin isotype. The characteristics of these fluorochrome conjugates have been reported previously³⁹. After a final wash in PBS, all preparations were fixed in 96% ethanol (15 min), rinsed in PBS, and mounted in a polyvinyl alcohol medium for fluorescence microscopy⁸.

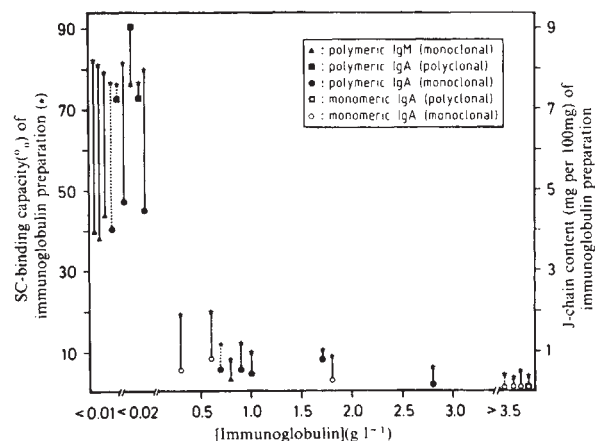


Fig. 2 Staining end points for binding of various human immunoglobulin preparations to the surface of HT29.E10 cells as determined by indirect immunofluorescence (see Fig. 1). The results were based on microscopic evaluation of 175 monolayer preparations incubated with IgA and 45 incubated with IgM. The abscissa gives the concentration of immunoglobulin needed for binding to SC-expressing epithelial cells. The plots of SC-binding capacity (*) and J-chain content (different symbols as indicated) are connected by vertical lines (broken for fluorescein-labelled IgA preparations); these data were obtained as described in Table 1.

Table 1 Characteristics of human immunoglobulin preparations used in binding studies with HT29.E10 cells

No. and type of preparation	Approximate composition	Molar <i>F/P</i> ratio	J-chain content (mg per 100 mg)	SC-binding capacity (%)*
1 Pc Pm IgA	78% Dimers 15% Larger polymers 7% Monomers	4.0	7.3	76
1 Pc Pm IgA	78% Dimers 15% Larger polymers 7% Monomers	None	7.3	76
2 Pc Pm IgA	70% Dimers 25% Larger polymers 5% Monomers	None	9.3	76
3 Mc Pm IgA	70% Dimers 20% Larger polymers 10% Monomers	0.9	4.0	77
4, 5 Mc Pm IgA	Mainly trimers 5–10% Monomers	None	4.5–4.7	80
6 Mc Pm IgA	20% Dimers 40% Trimers 40% Larger polymers	3.0	0.6	12
6 Mc Pm IgA	20% Dimers 40% Trimers 40% Larger polymers	None	0.6	12
7–9 Mc Pm IgA	40–60% Dimers 25–50% Larger polymers 10–20% Monomers	None	0.2–0.8	7–10
10 Colostral IgA	75% Dimers 25% Larger polymers	3.2	ND	19
11 Mc Mm IgA	Mainly monomers	3.6	0	4
12 Pc Mm IgA	Mainly monomers	None	0	4
13,14 Mc Mm IgA	Mainly monomers	None	0	4–5
15–17 Mc Mm IgA	Mainly monomers	None	0.3–0.8	9–20
18–20 Mc Pm IgM	Mainly pentamers	None	3.9–4.4	80–82
21 Mc Pm IgM	Mainly hexamers	None	0.5	8

Pc, polyclonal; Pm, polymeric; Mc, monoclonal; Mm, monomeric. Purification was achieved by anion-exchange chromatography and gel filtration⁶. It is unknown whether the J chain-deficient polymers had been produced as such or whether the J chain had been removed by selective proteolysis *in vivo* or *in vitro*³⁶. The fact that preparation 21 consisted mainly of hexamers³³ suggests the former possibility. The approximate composition of each preparation was determined by density gradient centrifugation⁶. The *F/P* (fluorochrome/protein) ratio was determined as described previously for preparations labelled with fluorescein isothiocyanate³⁷. The conjugation method and purification of the conjugates by anion-exchange chromatography followed standardized procedures³⁷. J-chain content was measured for reduced preparations by an immunochemical method described previously³⁸. ND, not determined.

* Percentage of 2.5 µg iodinated SC spontaneously associated with 100 µg protein as determined by density gradient centrifugation³.

of J chain and immunoglobulin occurs only in IgA and IgM cells, thereby generating functional SC-binding sites already at the intracellular level¹⁴. Several studies have shown that the cytoplasmic appearance of J chain depends on B-cell stimulation^{28,29}; clonal maturity may, on the other hand, result in repression of J chain synthesis³⁰. The immunocytes populating secretory tissues apparently belong to relatively immature memory clones³¹; this feature probably explains the fact that not only do more than 90% of their IgA- and IgM-producing cells express J chain but so do most of the IgD-producing cells and 50–70% of the IgG-producing cells at these sites¹⁴.

The obvious functional goal of J-chain expression at a certain stage of clonal B-cell differentiation is generation of SC-binding IgA and IgM locally where external transport through SC-positive epithelium can occur readily. However, it is not clear how the J chain is involved in the binding site; it may act solely by causing structural modification of the α and μ chains present in the immunoglobulin polymers. Studies both *in vitro* and *in vivo* have suggested that the J chain is not required absolutely for polymerization^{32–34}, but it clearly facilitates intracellular formation of selected polymer species that can combine with SC¹⁴. Despite the fact that purified dimeric J chain affords only marginal blocking of SC binding to dimeric IgA and pentameric IgM³⁵, the possibility of direct non-covalent interactions between polymer-associated J chain and SC cannot be excluded.

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Immunoglobulin κ light-chain diversity in rabbit is based on the 3' length heterogeneity of germ-line variable genes

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Antibody diversity is generated by the combinatorial association of multiple distinct genetic segments (variable (V), joining (J) and diversity (D) light (L) and heavy (H) chains— V_L , J_L and V_H , D , J_H) and amplified somatically by three or four different mechanisms¹⁻⁶. The κ system in mouse and human consists of 50–100 V_κ segments associated with a cluster of four or five functional J_κ segments, located 2.5 kilobases (kb) 5' to a single C_κ gene⁷⁻¹⁰. The third hypervariable region (CDR3), which is part of the antibody combining site, is usually nine amino acids long in human and mouse κ chains. It is encoded by the last seven codons of the V_κ segment and the first two of the J_κ segment, one codon sometimes being added or deleted between V and J by junctional variation². In the rabbit, the $C_\kappa 1$ gene which encodes the major isotype¹¹⁻¹⁴, is associated with a cluster of five J_κ segments, only one of which seems to be functional¹¹, thus significantly decreasing the combinatorial potential. However, amino acid sequence comparison has revealed extensive heterogeneity in the length of rabbit CDR3 (ref. 15), suggesting the existence of a D segment analogous to that in the heavy-chain system. We show here that rabbit V_κ genes have several additional nucleotides at their 3' ends. Thus, even with a single functional J_κ segment, high CDR3 diversity can be generated based on the length heterogeneity of V_κ germ-line segments and their greater length, which might leave scope for an increased junctional deletion.

We have isolated many recombinant cosmids from a rabbit liver genomic library¹³ and find that each DNA insert of ~35 kb contains four to six *EcoRI* fragments that hybridize to a V_κ probe; this suggests that rabbit V_κ genes are clustered and spaced 6–8 kb apart. Human and mouse V_κ genes, with few exceptions¹⁶, seem to be separated by 15 or 20 kb^{17,18}. By Southern genomic blot experiments (Fig. 1), a single V_κ probe can detect as many as 25–30 restriction fragments displaying different intensities, and reveals little restriction site polymorphism between four independent individuals. Compared with mouse and human, where a single V_κ probe detects, respectively, 1–15 and 15–20 restriction fragments^{19,20}, in the rabbit at least one group of cross-hybridizing V_κ genes is greatly amplified. However, V_κ subgroups are not clearly defined^{21,22}. It remains to be determined whether the probe can detect a large proportion of the V_κ gene repertoire, as in human, or only a small subset of this repertoire, as in mouse.

Figure 2 shows the nucleotide sequences of four unrearranged V_κ segments, isolated by pairs on two independent recombinant cosmids. They contain two exons, as in human and mouse^{23,24}, a small exon encoding most of the peptide leader sequence, separated by a 123-base pair (bp) intron from a large exon encoding the end of the signal peptide and the V_κ region. Whereas many human and mouse V_κ and V_H pseudogenes have been reported²³⁻²⁵, these four rabbit V_κ genes show apparently normal signal peptide and V_κ coding sequences. The deduced amino acid sequences are 85% homologous (apart from CDR3, see also Fig. 3), the most common amino acid sequence being that defined by Kabat *et al.*¹⁵. As in mouse and human where signal peptides are usually 20 or 22 amino acids long^{23,24}, the ATG codon (position 143 in Fig. 2) gives a 22 amino acid signal peptide. We also note, 30 bp upstream, another in-phase ATG codon giving rise to a 32 amino acid signal peptide²⁶. The promoter sequences (CAAT-like and TATA-like boxes) are

Table 1 Percentages of similarity between V_κ genes

	5'	P _L	Int	V _κ	3'
V18a/V18b		95.4 (90.9)	95.9	88.4 (79.2)	95.0
V19a/V19b	89.0	93.9 (90.9)	92.7	86.4 (80.6)	92.0
V18/V19	90–95	95–97 (91–100)	94–97	81–88 (71–81)	91–96
V18a/HK102	72.9	92.4 (81.8)	62.1	74.0 (61.0)	72.4

Nucleotide sequences of the 5' flanking (5'), leader peptide (P_L), intron (Int), V_κ coding (V_κ) and 3' flanking (3') regions were compared and percentages were calculated as: number of substitutions to number of nucleotides compared. Each deletion or insertion of nucleotide (whatever the number) was scored as a single mutation. Numbers in parentheses correspond to percentages of similarity at the amino acid level. Numbers given for 18/19 (lane 3) correspond to the minimum and maximum values obtained when a V18 gene (a or b) was compared with a V19 gene (a or b). Human V_κ HK102 is from ref. 23.

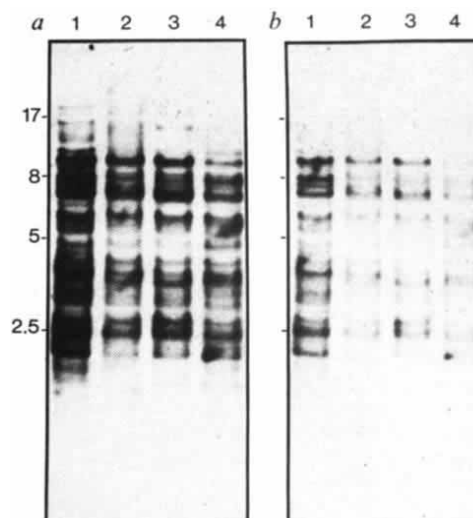
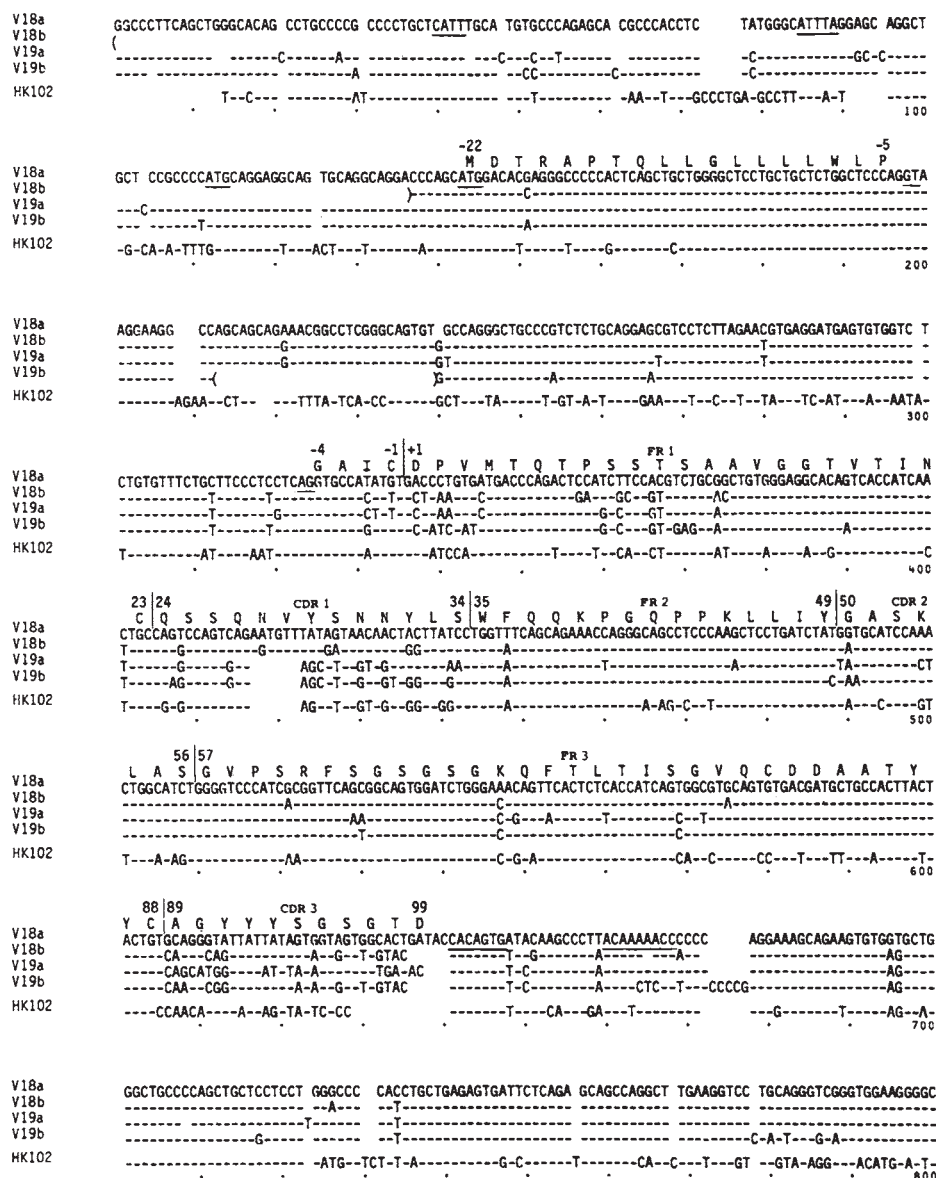


Fig. 1 Southern blot analysis of four individual rabbit DNAs using the V19a gene as a probe. Liver DNAs of homozygous *b9/b9* (lane 1), *b4/b4* (lane 2), *b5/b5* (lane 3) and *b6/b6* (lane 4) rabbits were digested with *EcoRI*, electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose³⁴. Hybridization was according to ref. 35. The probe was the 1.5 kb V19a *EcoRI* insert (see Fig. 2 legend); this was purified, ligated to itself and nick-translated, as already described³⁵. *a*, The blot was washed in low-stringency conditions, at 65 °C in 2 × SSC, 0.1% SDS for 90 min with three changes, and autoradiographed for 2 days. *b*, The same blot was then washed in high-stringency conditions, at 65 °C in 0.1 × SSC, 0.1% SDS for 30 min, and autoradiographed for 15 days.

located at the same distance from the ATG codon-22 as in the human HK102 V_κ gene (Fig. 2). Percentages of similarity given in Table 1 show that signal peptides are highly conserved and that rabbit flanking sequences and introns are more conserved than the V_κ coding regions. Furthermore, as shown in Fig. 2, base pair substitutions are preferentially clustered in the first third and in the CDR3 regions of the V_κ genes. Such patterns of segmental homology are highly characteristic of gene conversion events^{17,27-31}.

As in human and mouse, the recombination signal at the end of the V_κ coding sequence consists of the heptamer CACAGTG separated from the nonamer AAAAAAACC by a 12-bp non-conserved spacer¹. The signal sequences of V18a and V19a are perfectly conserved. V18b and V19b have, respectively, one base pair deletion and three base pair substitutions in the nonamer.

Fig. 2 DNA sequence comparison of four germ-line rabbit V_{κ} genes and the human HK102 V_{κ} gene²³. A rabbit $V_{\kappa} + C_{\kappa}$ cDNA probe was constructed from b5/b5 rabbit spleen mRNA, as previously described³⁶. A cosmid library of adult b4/b4 rabbit liver DNA fragments¹³ was screened by *in situ* hybridization with the nick-translated $V_{\kappa} + C_{\kappa}$ cDNA insert, as already reported¹³. Two recombinant cosmids, V4R-18 and V4R-19, were digested with *Eco*RI and analysed by Southern blot hybridization³⁴ with the cDNA probe. Two positive *Eco*RI restriction fragments were chosen from each cosmid (2.1 kb (V18a) and 2.9 kb (V18b) from V4R-18; 1.3 kb (V19a) and 2.2 kb (V19b) from V4R-19). *Sau*3A, *Hae*III or *Ava*II restriction digests of the four *Eco*RI fragments were subcloned into M13mp701 vector and sequenced on both strands by the chain-termination method³⁷⁻³⁹. The amino acid translation of the V18a gene is given in the one-letter code⁴⁰ and the numbering is according to ref. 15. The frameworks (FR) and complementarity-determining regions (CDR) are indicated. Dashes indicate nucleotide identities with the V18a nucleotide sequence. Blank spaces have been introduced to maximize homology. Parentheses in V18b and V19b genes correspond to unidentified residues, and alignments have been made in accordance with V18a gene and restriction mapping analysis. Promoter signal sequences, translational initiation codons, splicing signal and recombination recognition signal sequences are underlined.



Because the rabbit κ genes conform to the 12/23-bp rule, we can exclude the possibility that an intermediate *D* segment accounts for the high heterogeneity in CDR3 length. As shown in Fig. 2, the rabbit V_{κ} genes are very heterogeneous in length and sequence in their CDR3 regions, and are longer than in human and mouse. They vary from 30 to 35 bp and can code for 10 or 11 amino acids, whereas in human and mouse, CDR3 regions are usually 22–23 bp encoding 7 amino acids, with two exceptions being 25 bp long^{32,33}.

In fact, the rabbit CDR3 region has been shown to contain up to six more amino acids than human and mouse¹¹. However, in the two cases where there are six extra amino acids, the sixth (Thr or Gly) could be derived from the J_{κ} codon (Ala) by a single (somatic?) point mutation. The four rabbit V_{κ} germ-line genes in the present study can code for three or four extra amino acids, or four or five, depending on junctional variation². It is thus very likely from our data, that the extra amino acids found in the rabbit κ -chains are entirely encoded by the V_{κ} genes, without the need for an N region as in the heavy-chain system³.

Recombination between V_{κ} and J_{κ} segments may delete nucleotides in the V_{κ} genes, as in the J_{κ} segment. Junctional deletions have been observed mainly at the $V_{H}-D$ and $D-J_{H}$ junctions, with only rare exceptions in the light-chain system³². As the signal sequences involved in the $V_{\kappa}-J_{\kappa}$ junction are identical in rabbit and mouse or human, the junctional deletion

or variation must follow the strand cut during recombination. This deletion mechanism is probably of the same kind as in the heavy-chain system. It might have been selected in the rabbit because of structural constraints linked to the length of J_{κ} and V_{κ} segments. Thus, the additional nucleotides at the 3' end of rabbit V_{κ} genes are somewhat analogous to the *D* segment of the heavy-chain system. They leave scope for an increased junctional deletion mechanism during rearrangement, which in turn generates high CDR3 diversity.

Junctional deletion of the rabbit J_{κ} codons means that they are no longer involved in the formation of CDR3 (ref. 11) and thus J_{κ} segments can be inactivated during evolution, without serious consequences, provided, of course, that one J_{κ} segment remains functional. The rabbit V_{κ} genes may correspond to an ancestral system from which the human and mouse V_{κ} genes have been derived by nucleotide deletions during evolution of these species. In this respect, it is interesting that human λ light chains also show CDR3 length heterogeneity¹⁵, suggesting a possible common origin for the human λ and rabbit V_{κ} genes.

In conclusion, immunoglobulin genetic segments can to variable extents use the mechanisms available for the generation of antibody diversity. κ Light-chain CDR3 diversity in human and mouse is based on combinatorial potential and is amplified by a limited junctional variation mechanism. Our data show that in the rabbit only a single functional J_{κ} segment is required.

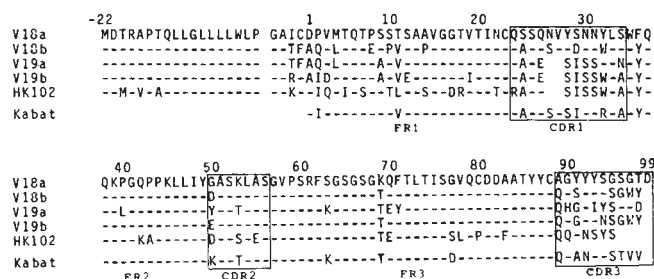


Fig. 3 Deduced amino acid sequences of the four rabbit germline V_{κ} genes. The one-letter code is used⁴⁰ and numbering is according to ref. 15. Dashes indicated identities with the amino acid sequence of the V18a gene. Gaps have been introduced to maximize homology. Also given is the deduced amino acid sequence of the human HK102 V_{κ} gene²³. Kabat's sequence is a compilation of rabbit κ light-chain amino acid sequences and represents for each residue the most common amino acid observed at that position¹⁵.

The diversity is based on the greater length and length heterogeneity of germ-line V_{κ} -CDR3 regions, and is highly amplified by nucleotide deletions in the J_{κ} and perhaps V_{κ} segments, during V-J recombination. Diversity in rabbit κ light chains mimics the mechanisms of CDR3 diversity in human and mouse heavy-chain systems, where V_H segments are highly conserved at their 3' ends, and heterogeneity in length and sequence is provided by the D segments, the N region and the junctional deletion.

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Transcription of plant defence genes in response to UV light or fungal elicitor

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The synthesis of secondary metabolites in response to stress conditions has been implicated as a major defence response of higher plants¹. Cell suspension cultures of parsley (*Petroselinum hortense*) have been studied extensively as a model system and shown to respond to UV irradiation and treatment with fungal elicitor by the synthesis of flavonoids and furanocoumarins, respectively^{2,3}. The induced synthesis of these compounds might afford the plant protection against the respective environmental hazards, because flavonoids strongly absorb UV light and furanocoumarins possess fungitoxic properties⁴. The production of each class of compound is preceded by the induction of groups of coordinately regulated enzymes^{2,5-8}, based on rapid changes in amounts and activities of the corresponding messenger RNAs^{9,10}. We now report that these changes are due to transient increases in the transcription rates of the respective genes, which indicates that gene activation has an important role in UV and disease resistance in higher plants.

The three enzymes of general phenylpropanoid metabolism (group I) are co-induced in cultured parsley cells with either the flavonoid glycoside pathway, comprising some 16 enzymes, (group II) or the less clearly defined furanocoumarin pathway (group III), depending on whether UV light or a pathogen-derived elicitor¹¹ is used as inducing agent^{2,4,6,8}. The effect of UV light is highly selective for the synthesis of flavonoids, whereas various metabolic changes are induced by the elicitor^{4,10}. Cloned complementary DNA copies of mRNAs coding for two enzymes of group I, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and 4-coumarate: CoA ligase (4CL; EC 6.2.1.12), and for chalcone synthase (CHS), a member of group II, have recently been used to show that the accumulation of flavonoids or furanocoumarins is associated with rapid increases in the respective mRNAs^{9,10}. The same cDNAs were used in the present study, where nuclei were isolated from UV-irradiated and elicitor-treated cells, and RNA synthesis was continued *in vitro* in the presence of ³²P-UTP. The radioactive RNA was then hybridized to the cDNA inserts bound to diazobenzoyloxymethyl (DBM) filters or to DNA-nitrocellulose blots. The amount of ³²P-UTP incorporated into a specific mRNA is taken as a measure of the polymerase activity associated with the respective gene at the specific time of nuclei isolation. Because it is unlikely that initiation of transcription takes place in these conditions¹², we are measuring only the completion of transcripts initiated *in vivo* before the nuclei were isolated.

Irradiation of the cell cultures with UV light for a standard 2.5-h period^{5,7} induced transient increases in the transcription rates of the genes coding for PAL, 4CL and CHS mRNAs. Figure 1 shows the results for PAL and CHS, confirming the previously observed small, but distinct, difference in the timing of induction of groups I and II^{4,5,10}. Changes in the rates of 4CL mRNA synthesis were similar to those obtained for PAL, but as expected⁷, the amounts of hybridizable material were much smaller and more difficult to determine accurately (~2 parts per million (p.p.m.) at the peak around 4 h after the onset of induction).

Similar results were obtained in both types of determination, DNA blot (Fig. 1a) and DBM filter (Fig. 1b) hybridizations. The CHS cDNA used here encompasses the entire coding sequence of the mRNA¹³ and contains an internal PstI site. On isolation of the cDNA insert from the PstI site of the cloning vector, plasmid pBR322, two fragments of 720 and 780 base pairs were obtained (Fig. 1a).

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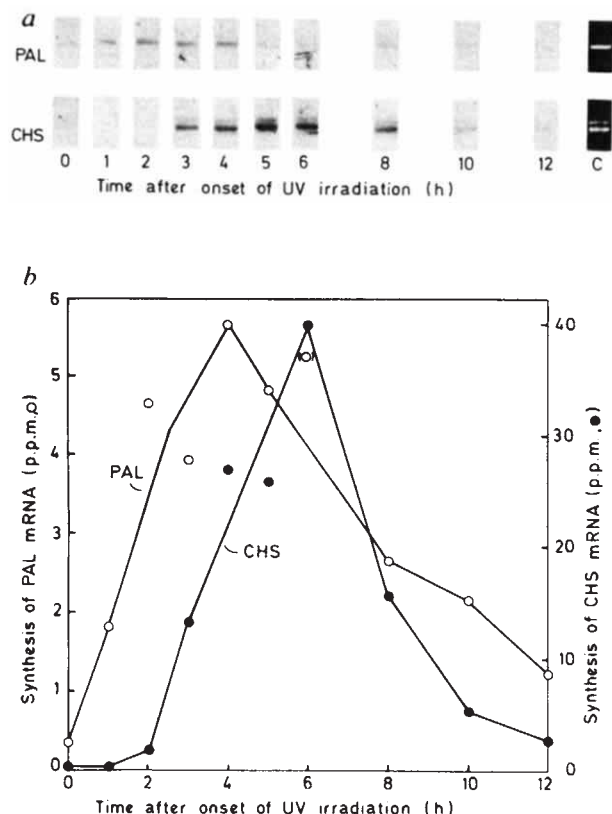


Fig. 1 Changes in the transcription rates of PAL and CHS genes in nuclei isolated from parsley cell cultures at various times after the onset of UV irradiation for 2.5 h. The 32 P-RNA synthesized by isolated nuclei was hybridized either to DNA blots (a) or to DBM filters (b) containing cDNA inserts complementary to PAL and CHS mRNAs. The amount of RNA hybridizing to a specific cDNA was determined by autoradiography (a) or by scintillation spectrometry (b). A control (C) shows the cDNA inserts stained with ethidium bromide.

Methods: A cell suspension culture of parsley (*Petroselinum hortense*) was grown in continuous darkness for 7 days after subculturing in a simple, synthetic medium⁷ and then irradiated with UV light^{5,7} for 2.5 h. Cells were collected at the indicated times after the onset of irradiation. Nuclei were isolated from the cells according to Willmitzer and Wagner¹⁴ with the following modifications. Cells (25–40 g) were gently stirred in 100 ml of 0.7 M mannitol, 10 mM methane ethane sulphonate (MES) pH 5.8, 5 mM EDTA, 0.1% bovine serum albumin (BSA) (w/v), 0.2 mM phenyl methylsulphonyl fluoride (PMSF) containing 50 mg cellulase (Onozuka) and 10 mg Macarzyme (Onozuka) for 15 min at 25 °C, and the partial protoplasmic cells were homogenized three times with an Ultra-turrax for 15 s in 0.25 M sucrose, 10 mM NaCl, 10 mM MES pH 6.0, 5 mM EDTA, 0.25 mM spermine-HCl, 0.5 mM spermidine-phosphate, 20 mM mercaptoethanol, 0.2 mM PMSF, 0.6% Triton X-100 (w/v) and 2% dextran (40,000) (w/v). In 100- μ l reaction volumes, 10⁷ isolated nuclei completed transcripts initiated *in vivo* in 75 mM (NH₄)₂SO₄, 8 mM MgCl₂, 0.5 mM each of GTP, ATP and CTP, 8% glycerol (v/v), 20 mM Tris-HCl pH 7.8 and 100 μ Ci 32 P-UTP (400 Ci mmol⁻¹) for 30 min at 30 °C. The reaction was linear in these conditions for about 60 min. The RNA synthesized *in vitro* was isolated¹⁵ and dissolved in 50% formamide, 4 $\times$ SSPE, 0.02% each of Ficoll, polyvinylpyrrolidone and BSA (w/v), 200 μ g ml⁻¹ each of transfer RNA and poly(A)⁺ RNA, and 0.2% SDS (w/v). The radiolabelled RNA (2 $\times$ 10⁷ c.p.m. per sample) was then hybridized to 200 ng insert DNA transferred to GeneScreen (NEN) filters (a) or to DBM filters¹⁶ containing 200 ng insert DNA (b). Hybridization was done in the same buffer containing 8% dextran sulphate (w/v) at 42 °C for 40–62 h. The filters were then washed three times with 2 $\times$ SSC, 0.5% SDS at 60 °C for 30 min, once with 0.5 $\times$ SSC, 0.5% SDS at 60 °C for 30 min, twice with 2 $\times$ SSC and subsequently treated with 20 μ g ml⁻¹ RNase A in the same buffer for 30 min at room temperature. The filters were finally washed twice in 2 $\times$ SSC with 0.5% SDS and twice in the same buffer without SDS. Additional amounts of DNA did not increase the amount of RNA hybridization, and a linear relationship existed between the amount of RNA used and RNA hybridization. The 32 P-RNA hybridized was visualized by autoradiography of the DNA blots (a) or quantified by scintillation spectrometry of the radioactivity released by treating the DBM filter with 0.4 M NaOH (b). The lanes on the far right in a show the ethidium bromide-stained agarose gel before transfer of the cDNA insert to GeneScreen (NEN). Hybridization to DBM filters was corrected for background by subtracting the radioactivity released from a filter containing 200 ng λ dV DNA. Specific hybridization is presented as p.p.m. of input RNA. 3 $\times$ 10⁷ c.p.m. of 32 P-RNA was used for each hybridization shown and the background hybridization was an average of 112 c.p.m. No corrections were made for the hybridization efficiency or size of the hybridization probe.

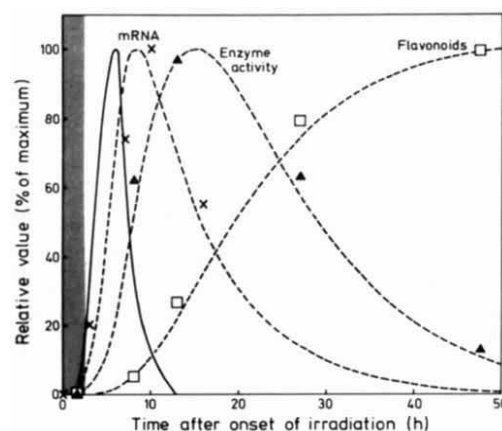


Fig. 2 Comparison of predicted and measured changes in CHS mRNA and enzyme activities and in flavonoid glycoside concentrations in UV-irradiated parsley cells. The shaded area indicates the length of the UV irradiation period. The solid curve represents the data shown in Fig. 1 for UV light-induced CHS gene transcription; this curve was used to calculate (broken curves) the expected changes in CHS mRNA and enzyme activity, and the accumulation of flavonoid glycosides, in the previously established conditions in which both the mRNA and the enzyme were rapidly degraded⁵ but the flavonoids were not further metabolized¹⁷. In these conditions, the equation

$$dx(t)/dt = {}^0k_1(t) - {}^1k_d x(t) \quad (1)$$

can be applied to calculate mRNA or enzyme activity changes (x) with time (t) from the respective zero-order rate constants of synthesis (0k_1) and the first-order rate constants of degradation (1k_d), and the equation

$$P(t) = \int_0^t E(\tau) d\tau \quad (2)$$

describes the changes in the amount of products (P) accumulated with time (t, τ) as a result of changes in the rate-limiting enzyme activity (E)^{4,5,18}. The symbols represent mean values of two measurements each of CHS mRNA (x), CHS activity (Δ) and total flavonoids ($\square$). Changes in levels of CHS mRNA and translational activity have previously been shown to follow the same kinetics^{9,10}.

Methods: Propagation and collection of cells at the indicated times, extraction procedures and measurements of hybridizable mRNA amounts^{9,10} enzyme activity¹⁹ and amounts of total flavone and flavonol glycosides²⁰ have been reported previously.

The apparent changes in the rates of transcription were not due to an attenuation of the transcription rates of particular genes in control cells, because the same results were obtained in conditions where the nuclei had been lysed and the DNA superstructure disrupted by high salt concentrations (data not shown). Furthermore, the transcription rate of a constitutively expressed mRNA, which is complementary to cloned cDNA from the plasmid pLF14 and codes for a protein of unknown function (J.C. and K.H., unpublished results), was constant throughout the experiment (Fig. 1) at ~20 p.p.m. Ribosomal RNA synthesis, measured using a cloned maize genomic fragment comprising a cluster of ribosomal genes (P. Schulze-Lefert and G. Feix, manuscript in preparation), showed an approximate doubling over controls with 4 h. The *in vitro* transcription of the PAL, 4CL and CHS genes was sensitive to 0.1–0.4 μ g ml⁻¹ α -amanitin, suggesting that these genes are transcribed by RNA polymerase II.

The UV light-induced changes in the rates of PAL and CHS mRNA synthesis have also been measured *in vivo* (Table 1). Both mRNAs were synthesized at much higher rates 3–4 h after the onset of UV irradiation than in control cells or at a later stage after induction. These changes were basically the same as those measured *in vitro* and further demonstrate that the PAL and CHS genes are under transcriptional control in UV-irradiated cells.

Figure 2 shows the causal relationship between the timing of changes in the rate of CHS mRNA synthesis and the kinetics of flavonoid accumulation. The timing of the entire sequence of events, as predicted from the induced changes in the rates of CHS gene transcription and the rates of mRNA and enzyme degradation (which are easily calculated from the respective half lives^{4,5}), agrees with corresponding experimental results. Only a few time points are shown here for the changes in the

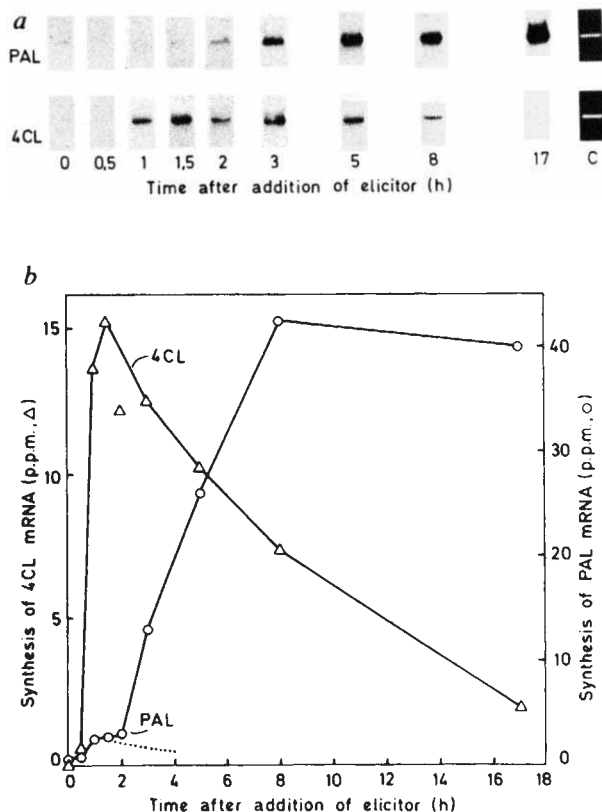


Fig. 3 Changes in the transcription rates of PAL and 4CL genes in nuclei isolated from cultured parsley cells at various times after the addition of elicitor. The 32 P-RNA synthesized by isolated nuclei was hybridized either to DNA blots (a) or to DBM filters (b) containing cDNA inserts complementary to the respective mRNA. The amount of RNA hybridizing to a specific cDNA was determined by autoradiography (a) or by scintillation spectrometry (b). A control (C) shows the cDNA inserts stained with ethidium bromide. The dotted line in b represents possible alternative changes in the rate of PAL mRNA synthesis (see text for discussion).

Methods: A cell suspension culture of parsley was treated at time 0 with $\sim 25 \mu\text{g ml}^{-1}$ of a fungal elicitor prepared from the cell wall of *Phytophthora megasperma* f. sp. *glycinea*¹¹. Culture conditions, nuclei isolation, RNA synthesis and hybridizations with PAL and 4CL cDNAs were as described for Fig. 1, except that 1.5×10^7 c.p.m. per hybridization were used. Specific hybridization, obtained by correction for an average background of 178 c.p.m., is expressed as p.p.m. of input RNA. No corrections for hybridization efficiency or the size of the hybridization probe were made.

amount of hybridizable mRNA and in enzyme activity, but a more detailed analysis has been reported previously^{5,9,10}. We conclude from the coincidence of the calculated and the experimentally derived data that the timing of flavonoid glycoside accumulation in UV-irradiated parsley cells is determined solely, or at least predominantly, by the kinetics of gene transcription. It is impossible, however, to deduce from our present data whether one particular enzyme of the two UV light-induced pathways is rate-limiting. Although PAL transcription follows different kinetics from CHS transcription, the apparent half lives of PAL and CHS mRNA and enzyme activities are also different⁵, resulting in a similar flavonoid accumulation curve when PAL rather than CHS is used for the calculations described above.

We have recently shown that either the same or very similar PAL and 4CL mRNAs were induced by treatment of parsley cells with UV light or with a fungal elicitor¹⁰. We therefore measured the transcription rates *in vitro* of the PAL and 4CL genes in nuclei isolated from elicitor-treated cells (Fig. 3). In agreement with observations at the level of changes in amounts of mRNA¹⁰, apparent differential rates of PAL and 4CL gene transcription in elicitor-treated cells were observed, and the elicitor-induced changes in the transcription rate of the 4CL gene were transient but more rapid than those induced by UV irradiation. The transcription rate of a mRNA constitutively expressed in other conditions and monitored with the cDNA

Table 1 UV light-induced *in vivo* PAL and CHS mRNA

Time of labelling	Total RNA used (c.p.m.)	PAL		CHS	
		c.p.m.*	p.p.m.	c.p.m.*	p.p.m.
-1-0 h	430,000	4	9	1	0
3-4 h	560,000	17	30	76	136
8-9 h	410,000	6	15	16	39

At the indicated times before or after the onset of 2.5 h UV irradiation, cell cultures were concentrated from 5 g per 40 ml to 5 g per 5 ml medium by gentle filtration and labelled for 1 h with 0.5 mCi each of [5,6- ^3H]uridine (40-60 Ci mmol⁻¹), [5- ^3H]cytosine (25-30 Ci mmol⁻¹) and [5- ^3H]guanosine (20-40 Ci mmol⁻¹). The cells were collected by suction filtration, washed with sterile water and frozen in liquid nitrogen. Polyribosomal RNA and poly(A)⁺ RNA were isolated from the cells as described previously⁹. The poly(A)⁺ RNA (specific activity 35,000-70,000 c.p.m. per μg RNA) was hybridized to DBM filters containing 200 ng of the appropriate cDNA insert. Hybridization, washes and release of selected RNAs were as described in Fig. 1 legend. Specific hybridization was determined by subtracting background hybridization to a filter containing 200 ng of λ dV DNA, which gave a mean value of 15 c.p.m. Specific hybridization is also presented as p.p.m. of the input RNA. dV = defective virulent.

* Corrected for non-specific hybridization.

insert from pLF14 (see above) declined slowly after elicitor treatment from 20 p.p.m. to 10 p.p.m. within the period of the experiment. Flavonoids are not synthesized in elicitor-treated cells, and the elicitor has no effect on CHS⁶.

The relatively high rate of 4CL gene transcription and the low initial rate of PAL gene transcription in elicitor-treated cells, as compared with UV-irradiated cells, are consistent with similar relative levels of both the enzyme activities and the mRNA levels^{6,10}. As discussed elsewhere¹⁰, the apparent late and large increase in the transcription rate of the PAL gene and the corresponding accumulation of PAL, or PAL-like, mRNA in elicitor-treated cells are unexpected in view of the rapid, transient changes in the translational activity of PAL mRNA and might be due in part to cross-hybridization of the PAL cDNA with other elicitor-induced mRNA(s) of similar size. The actual changes in the rate of PAL gene transcription responsible for the coordination of the PAL and 4CL mRNA and enzyme activity changes^{6,10} might follow a time course similar to that indicated by the dotted line in Fig. 3. Experiments are in progress to clarify this point. In any case, we have shown here for the first time that both types of stress, UV light or pathogen-derived elicitor, induce the respective defence response of the challenged plant cells by transcriptional control of genes involved in the biosynthesis of resistance-related compounds.

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Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase

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The initiation of protein synthesis can be regulated in mammalian cells by protein kinases which phosphorylate the α subunit of initiation factor eIF-2 (ref. 1). This phosphorylation results in a block in the recycling of eIF-2 (refs 2, 3) and in the inhibition of messenger RNA binding to 80S initiation complexes^{4,5}. After eIF-2 α is phosphorylated, the mRNA becomes associated with 48S complexes consisting of a 40S ribosomal subunit⁴, eIF-2(α P), GDP and Met-tRNA_i (ref. 5). One of the eIF-2 α kinases is activated by low concentrations of double-stranded RNA (dsRNA)⁶. This kinase (PK_{ds}) is present at a basal level in all mammalian cells investigated⁷ and its synthesis is induced in cells treated with interferon⁸. The PK_{ds} may be involved in the inhibition of translation of viral mRNA in interferon-treated cells infected with RNA viruses, as it is activated by viral replicative complexes. It is not known, however, if the activated PK_{ds} preferentially inhibits the translation of viral mRNA when cellular protein synthesis proceeds at a normal rate in infected cells⁹. We now report that mRNA covalently linked to dsRNA is preferentially inhibited from binding to 80S complexes by a localized activation of PK_{ds}. This suggests that in interferon-treated cells the binding of some nascent viral mRNAs to functional initiation complexes may be preferentially inhibited by a similar mechanism.

The initial experiments were carried out with ³H-uridine-labelled mRNA from vesicular stomatitis virus (VSV). This was purified by absorption to nitrocellulose filters to obtain mRNA with poly(A) sequences longer than 60 nucleotides¹⁰. This mRNA was incubated with reticulocyte lysate to determine its binding to initiation complexes, which were fractionated by centrifugation on sucrose gradients⁵. After incubation at 0 °C, unbound mRNA sedimented more slowly than 40S ribosomal subunits (Fig. 1a), whereas after incubation at 30 °C the mRNA was quantitatively bound to 80S initiation complexes (Fig. 1b). Further addition of ribosomes to 80S complexes was prevented by the inclusion of emetine, an inhibitor of elongation⁵. When the lysate was incubated with 750 ng ml⁻¹ of the dsRNA poly(A)·poly(U) for 7 min before the addition of VSV mRNA, the assembly of 80S complexes was inhibited and the mRNA sedimented instead at 48S (Fig. 1c). When the mRNA and dsRNA were added together, however, most of the mRNA bound to 80S complexes (Fig. 1d). This finding could be explained by the observation that it takes up to 5 min after the addition of dsRNA to obtain extensive phosphorylation of eIF-2 (refs 5, 11). The mRNA could bind to 80S complexes within 90 s¹², before a significant fraction of eIF-2 was phosphorylated.

A completely different result was obtained when the dsRNA was covalently linked to mRNA. This dsRNA was formed by annealing poly(U) to the poly(A) sequence of VSV mRNA, as previously described¹³, to form dsRNA longer than 60 base pairs which can activate the PK_{ds} (ref. 14). The mRNA containing poly(A)·poly(U) did not bind to 80S initiation complexes, but sedimented instead at 48S (Fig. 1e). In this experiment, the poly(A)·poly(U) linked to mRNA was present at the same concentration as 'free' poly(A)·poly(U) in the experiment shown in Fig. 1d. The dsRNA linked to mRNA was apparently responsible for inhibiting its binding to 80S initiation complexes. This may have been due to a localized activation of the eIF-2 kinase by the poly(A)·poly(U) in mRNA and the phosphorylation of a fraction of eIF-2 which became associated with this mRNA in 48S complexes. In support of this explanation, when 2-aminopurine, an inhibitor of the eIF-2 α kinase^{5,6} was added to a binding assay of mRNA containing the poly(A)·poly(U)

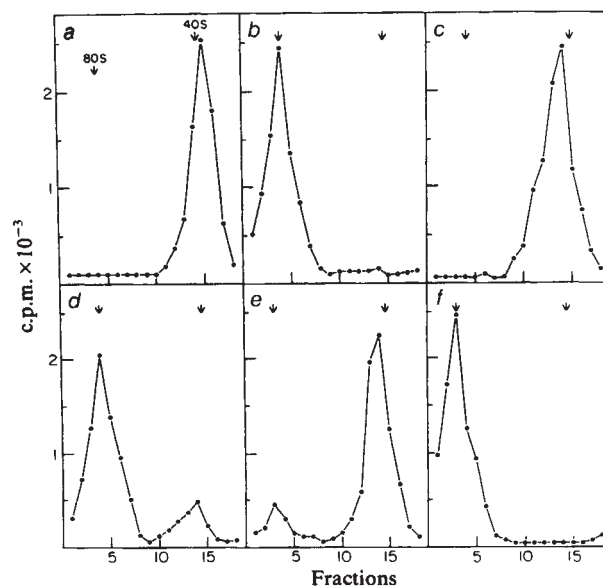


Fig. 1 Binding of VSV mRNA to initiation complexes. *a*, Incubation was at 0 °C. All the other incubations were at 30 °C and were stopped 3 min after the addition of mRNA. The ³H-mRNA was added after a 7-min preincubation without (*b*) or with 750 ng ml⁻¹ of poly(A)·poly(U) (*c*). This dsRNA was added together with ³H-mRNA in *d*. The mRNA contained a poly(A)·poly(U) duplex in *e* and *f*; 10 mM 2-aminopurine was added in *f*. The position of the 80S and 40S peaks is indicated by arrows. The VSV mRNA was labelled with ³H-uridine in infected cells, fractionated to obtain a 12–16S fraction⁵ and absorbed to nitrocellulose filters to select mRNA with poly(A) sequences longer than 60 nucleotides¹⁰. This mRNA was either directly used in binding assays or annealed with sufficient poly(U) to prevent its retention by oligo(dT)–cellulose¹³. The average size of the poly(U) (purchased from Miles Biochemicals) was 120 nucleotides. The binding assays were carried out in 0.1 ml reactions which contained 60 μ l of reticulocyte lysate supplemented with 50 μ M haemin, 10,000 c.p.m. of ³H-mRNA, 0.1 mM emetine and the other additions described elsewhere⁵. At the end of the incubation the assays were diluted with 3 vol of 0.5 M NaCl, 30 mM Mg(OAc)₂ and 20 mM Tris, pH 7.6 (buffer A) and centrifuged for 5 h at 38,000 r.p.m. through 12-ml gradients of 15–30% sucrose in buffer A. The gradient fractions were counted directly⁵.

duplex, the mRNA bound quantitatively to 80S complexes (Fig. 1f).

Figure 2 presents direct evidence for the activation of eIF-2 α kinase by dsRNA linked to mRNA. Lysate was incubated with [γ -³²P]ATP and 750 ng ml⁻¹ of free poly(A)·poly(U) or mRNA-linked poly(A)·poly(U). With no added dsRNA or after 3 min incubation with free poly(A)·poly(U), there was no detectable eIF-2 α phosphorylation (Fig. 2a, b). After 7 min of incubation with poly(A)·poly(U) or 3 min incubation with mRNA-linked poly(A)·poly(U), eIF-2 α was clearly phosphorylated (Fig. 2c, d). When 48S complexes were isolated and analysed by gel electrophoresis⁵, some eIF-2(α P) was found associated with these complexes (Fig. 2e, f). It appeared that eIF-2 α was phosphorylated at a faster rate with mRNA-linked poly(A)·poly(U) than with free poly(A)·poly(U). This could be explained by a slow rate of dephosphorylation of eIF-2(α P) bound to 48S complexes relative to unbound eIF-2(α P)¹⁵. In one incubation, the PK_{ds} bound to mRNA-linked poly(A)·poly(U) preferentially phosphorylated eIF-2 α associated with 48S complexes, whereas in the other the PK_{ds} bound to free poly(A)·poly(U) phosphorylated eIF-2 α which could be readily dephosphorylated by lysate phosphatases¹⁵. In fact, a 70,000 molecular weight polypeptide previously identified¹⁶ with PK_{ds} was detected in 48S complexes isolated from the incubation with mRNA-linked poly(A)·poly(U) (Fig. 2f). This polypeptide could represent PK_{ds} bound to the 3'-terminal duplex of mRNA in these complexes.

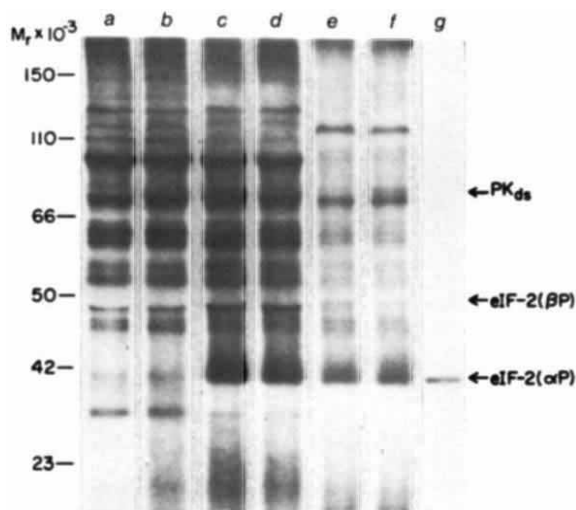


Fig. 2 Electrophoretic analysis of the phosphoproteins labelled: *a*, in 7 min incubation with no added dsRNA; *b*, *c*, in 3 and 7 min with 750 ng ml⁻¹ of poly(A)·poly(U); *d*, in 3 min with mRNA annealed to poly(U) as described in Fig. 1*e* (750 ng ml⁻¹ of dsRNA). The incubations were assembled as in Fig. 1, except that ATP was substituted with 10 μ Ci of [γ -³²P]ATP; 20 μ l of each reaction were directly analysed (*a*–*d*), whereas 80 μ l of the incubations shown in *c* and *d* were fractionated by gradient sedimentation to isolate the 48S peak. Gradient fractions were combined and proteins precipitated with 2 vol of acetone (*e*, *f*). The proteins were fractionated on 10% polyacrylamide–0.26% bisacrylamide gels. Authentic eIF-2 phosphorylated in a separate reaction is shown in *g*. To activate eIF-2 kinase, reticulocyte lysate was incubated for 15 min without added haemin⁶; 3 μ l of this lysate were incubated for 4 min with 2 μ g of purified eIF-2 (a gift of Dr William C. Merrick), 50 μ M (γ -³²P]ATP (1 μ Ci), 15 μ g of bovine serum albumin and 1.5 mM Mg(OAc)₂. 0.2 μ g of eIF-2 were analysed. The position of the PK_{d8}, eIF-2(α P), eIF-2(β P) and molecular weight markers is indicated.

In subsequent experiments, we demonstrated a preferential inhibition of the initiation of mRNA containing a poly(A)·poly(U) duplex by including in the same incubation mRNA not annealed with poly(A)·poly(U) and labelled with a different isotope. In these experiments, poly(U) was labelled to high specific activity with terminal transferase¹⁷ and annealed with unlabelled HeLa cell mRNA (Fig. 3). Only the mRNA containing a poly(A)·poly(U) duplex was thus labelled with ³²P. Very small amounts of this mRNA were added to binding assays together with uniformly ³H-labelled HeLa mRNA. The concentration of added dsRNA was reduced to 3 ng ml⁻¹ to delay the generalized inhibition of initiation observed in the experiment shown in Fig. 1*c*. Therefore, we carried out the binding assays without added emetine and allowed multiple cycles of initiation to take place. When an incubation was kept at 0°C, the two mRNAs sedimented more slowly than 40S ribosomal subunits (Fig. 3*a*). The ³H-mRNA was bound to 80S initiation complexes and to polyribosomes after 2–8 min incubation at 30°C, whereas the mRNA containing the poly(A)·poly(U) duplex sedimented predominantly at 48S (Fig. 2*b*–*d*). The ratio of ³H-mRNA bound to polyribosomes and 80S complexes to that sedimenting at 48S was always much greater than that of the ³²P-mRNA with the poly(A)·poly(U) duplex. It should be pointed out that even with as little as 3 ng ml⁻¹ of dsRNA, the initiation of protein synthesis was inhibited after 8 min of incubation⁶ and a significant fraction of ³H-mRNA sedimented in 48S complexes.

To demonstrate that the inhibition of binding of this mRNA was caused by the activation of PK_{d8}, a binding assay was performed using a high concentration of added dsRNA, thus specifically preventing the activation of this kinase⁶. When HeLa cell mRNA annealed with labelled poly(U) was incubated with lysate, its binding to 80S initiation complexes was inhibited (Fig.

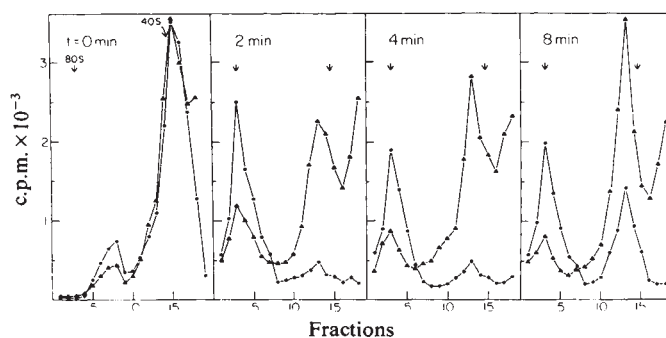


Fig. 3 Binding of HeLa cell mRNA to initiation complexes. Each 0.1-ml binding assay contained 17,500 c.p.m. of ³H-mRNA (●) and ³²P-mRNA (▲) with a poly(A)·poly(U) duplex. The reactions were incubated at 30°C for the time indicated in the panel and analysed as described in Fig. 1 legend, except that the gradient pellets containing polyribosomes were also counted. The ratio of c.p.m. in polyribosomes and 80S initiation complexes over c.p.m. in 48S complexes was 8.6, 6.7 and 2.5 for the ³H-mRNA, and 0.7, 0.4 and 0.3 for the ³²P-mRNA at 2, 4 and 8 min, respectively. The ³H-mRNA (58,000 c.p.m. per μ g) was obtained from HeLa cells labelled with ³H-uridine¹³ and the ³²P-mRNA by annealing 200 ng of unlabelled HeLa cells mRNA with 2 ng of poly(U) labelled by 3'-terminal addition of [γ -³²P]dATP. In a 2- μ l reaction, 200 ng of poly(U) were incubated for 4 h with 50 μ M (α -³²P]dATP (150 μ Ci), 2.5 U of calf thymus deoxynucleotide terminal transferase and 0.5 μ g of bovine serum albumin in 0.2 M K-cacodylate, 2 mM dithiothreitol, 0.5 mM CoCl₂ and 25 mM Tris, pH 6.9 (ref. 17). The reaction was stopped by the addition of 0.1 ml of NaOAc buffer, pH 5.2, containing 5 μ g of yeast tRNA. The RNA was precipitated with 0.4 ml of 3% cetyltrimethylammonium bromide, washed with this solution and 75% ethanol, and dissolved in 0.125 M KOAc and 20 mM HEPES buffer, pH 7.4. The poly(U) had a specific activity of 1.3×10^5 c.p.m. per ng and the specific activity of the mRNA annealed with this poly(U) was $\sim 1 \times 10^4$ c.p.m. per ng.

4*a*). With 10 μ g ml⁻¹ of poly(I)·poly(C) present, however, this mRNA was quantitatively bound to 80S complexes (Fig. 4*b*). Two other experiments provided additional evidence that the inhibition of binding of mRNA covalently linked to dsRNA required the activation of PK_{d8}. A mRNA containing a hybrid poly(A)·poly(dT) duplex was quantitatively bound to 80S initiation complexes (Fig. 4*c*). This showed that an RNA/DNA duplex, which could not activate the PK_{d8} (ref. 18), had no effect on mRNA binding. Another experiment, based on the observation that 50 μ M ethidium bromide prevented the activation of PK_{d8} by poly(A)·poly(U) (ref. 19), showed that the inhibition of binding of mRNA with the poly(A)·poly(U) duplex was largely abolished by adding this concentration of ethidium bromide to a binding assay (Fig. 4*d*).

The inhibition of binding of mRNAs covalently linked to dsRNA was discovered with model polynucleotides constructed for the present experiments. The mechanism involved in this inhibition, however, is of general interest as it may function in interferon-treated cells preferentially to inhibit translation of viral mRNA. This mechanism involves the activation of PK_{d8}, the phosphorylation of eIF-2 α and the binding of mRNA and eIF-2(α P) to 48S complexes. In the experiments with two mRNAs labelled with different isotopes present in the same incubation, only the binding of mRNA linked to dsRNA was inhibited. Therefore, eIF-2(α P) presumably associated with mRNA close to the site of activation of PK_{d8}. This could result from the binding of this kinase to the mRNA-linked poly(A)·poly(U) and the phosphorylation of eIF-2 interacting with this mRNA during the formation of 48S initiation complexes. The association of eIF-2 with specific nucleotide sequences near the 5'-terminus of mRNA has been reported²⁰, but it is not known whether these sequences interact with eIF-2 already bound to 40S ribosomal subunits during the assembly of initiation complexes.

On addition of 2-aminopurine and dephosphorylation of eIF-

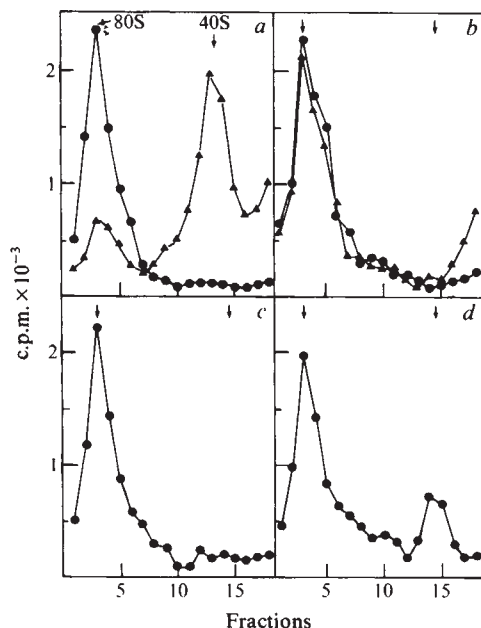


Fig. 4 Binding of HeLa cell mRNA to initiation complexes. The binding was carried out for 3 min after the addition of mRNA and analysed as described in Fig. 1 legend. The assays were incubated for 7 min without (a) or with (b) $10 \mu\text{g ml}^{-1}$ of poly(I)·poly(C) before the addition of 10,000 c.p.m. of HeLa cell ^3H -mRNA (●) and of mRNA containing a poly(A)·(U) duplex labelled with ^{32}P (▲) as described in Fig. 3 legend. The other assays contained mRNA with a poly(A)·poly(dT) duplex (c) or with a poly(A)·poly(U) duplex (d). Poly(dT) and poly(U) were labelled with terminal transferase as described in Fig. 3 legend, except that $2 \mu\text{g}$ of polynucleotide and $10 \mu\text{Ci}$ of ^3H -dCTP were used. The polynucleotides were labelled to a specific activity of 5×10^3 c.p.m. per ng and 10 ng were annealed with $1 \mu\text{g}$ of HeLa cell mRNA. $50 \mu\text{M}$ ethidium bromide was added to the binding assay in d.

$2(\alpha\text{P})$ by phosphatases²¹, the mRNA binds slowly to 80S initiation complexes⁵, indicating that mRNA in 48S complexes is not inactivated. It seems possible that mRNA synthesized by viral replicative complexes which activate the PK_{ds} becomes bound to 48S complexes. Comparison of the rate of cleavage of mRNA in polyribosomes with that of mRNA associated with 48S complexes shows that the latter is cleaved significantly faster by the endonuclease activated by 2',5'-oligo(A) (ref. 15). As these oligonucleotides may be synthesized by an interferon-induced enzyme bound to viral replicative complexes²² and can activate the endonuclease in a localized way¹³, it seems possible that mRNA in 48S complexes formed during transcription of viral templates is preferentially degraded. Experiments are in progress to investigate this in intact cells. It is also conceivable that PK_{ds} is involved in translational control of cellular mRNA if duplexes which can activate this kinase are formed.

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Activating protein factor binds *in vitro* to upstream control sequences in heat shock gene chromatin

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DNA sequences, important for the control of *Drosophila* heat shock gene expression, are packaged in chromatin in a nuclease hypersensitive configuration¹. Recently, two protein-binding (exonuclease-resistant) sites which cover the TATA box sequence and an upstream control element were shown to occur *in vivo* amidst the 5' terminal hypersensitive regions of several heat shock genes². Protein-binding at the TATA box is independent of heat shock, but the binding at the upstream element is heat shock dependent, and it was proposed that a heat shock activator protein, HAP, positively regulates the genes². Here, I report the detection of HAP activity in heat shocked cell extracts by reconstituting specific binding to *hsp82* gene chromatin *in vitro*. Inhibition of the binding by free DNA from the 5' region of heat shock genes implies a coordinate regulation of the gene family through HAP interaction with the upstream heat shock consensus sequence³. Furthermore, the special ease of induction of the *hsp82* gene over other heat shock genes can be explained in molecular terms by the higher affinity of HAP for the *hsp82* binding site, which contains a 28 base sequence with almost perfect dyad symmetry, GAAGCCTCTAGAAG|TTTCTAGAGACTTC.

We reasoned that promoter DNA in its naturally 'hypersensitive' state in chromatin should be a superior substrate to purified DNA for the *in vitro* binding of specific protein factors extracted from *Drosophila* cell nuclei. The binding to the target in chromatin can be revealed by the resistance to exonuclease digestion starting at an appropriate restriction endonuclease cut in the 5' hypersensitive region. We chose to monitor the binding activity of heat shocked cell extracts on the single-copy gene encoding the heat shock protein of molecular weight 81,858 (ref. 4–8) (*hsp82*; formerly referred to as *hsp83*), because of the exceptionally strong resistance to exonuclease digestion observed previously *in vivo*²; for experimental strategy, see Fig. 1.

The *in vivo* binding of HAP factor to the *hsp82* gene promoter in heat shocked *Drosophila* embryos is shown in Fig. 2. Purified nuclei from normal (lane a) or heat shocked (lanes b, c) embryos were digested with *EcoRI* and an exhaustive amount of *Escherichia coli* exonuclease III (exoIII). (ExoIII is unable to proceed with digestion from fragment ends made by those restriction enzymes, for example *PstI*, which leave a 4-base, 3' protrusion; S. Henikoff, personal communication.) DNA was purified and the single-stranded DNA tails were trimmed with *S₁* nuclease. After repurification, the DNA was cut with two restriction enzymes, *XhoI* and *HindIII*, and was sized by electrophoresis on agarose gels. The *hsp82* gene fragments resulting from *EcoRI* and exonuclease digestion were visualized with an indirect end-labelling probe after Southern transfer. Thus, the barrier to exonuclease digestion downstream from the *EcoRI* cut in the active gene was located at position –86, the distal border of the HAP binding site (Fig. 2, lanes b, c) whilst that

Fig. 1 The *in vitro*, chromatin binding assay. The nuclease hypersensitive site in the noninduced *hsp82* gene 5' region is represented as a stretch of DNA associated with the TATA box binding protein, (TAB)². Circles represent neighbouring nucleosomes whose precise positions have not been determined. HAP (+) and HAP (-) refer to the heat shocked and nonshocked condition, respectively.

at position -39 in the inactive gene reveals a boundary of the TATA box binding protein (TAB) (lane a)².

To assay for HAP factor *in vitro*, nuclei purified from non-heat-shocked *Drosophila* embryos were incubated with a 0.4 M NaCl extract of nuclei from heat-shocked embryos or cultured cells (Schneider line 2, SL2). After incubation with the extract, the nuclei were pelleted and resuspended for digestion with *Eco*RI and *exo*III, and the exonuclease-resistant sites were mapped as above. When using buffer alone, or an extract from non-heat-shocked embryos, or when histone H1 protein was used for the incubation, no resistance at position -86 was observed. However, distinct resistance at -86 was observed using extracts from heat-shocked embryos, and from two preparations of shocked SL2 cells. We are thus able to detect HAP binding *in vitro* using crude nuclear extracts without having to resort to previous purification.

HAP binding activity in shocked embryonic extracts is low compared to that observed in shocked SL2 cell extracts; this is

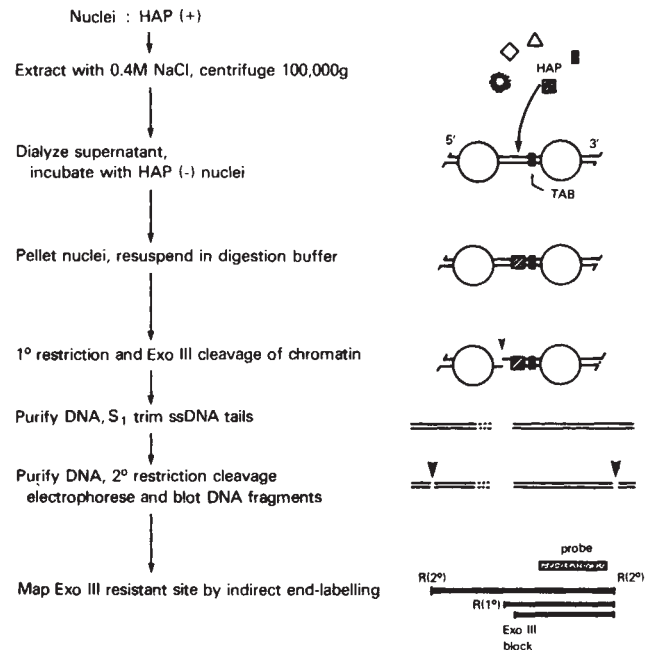
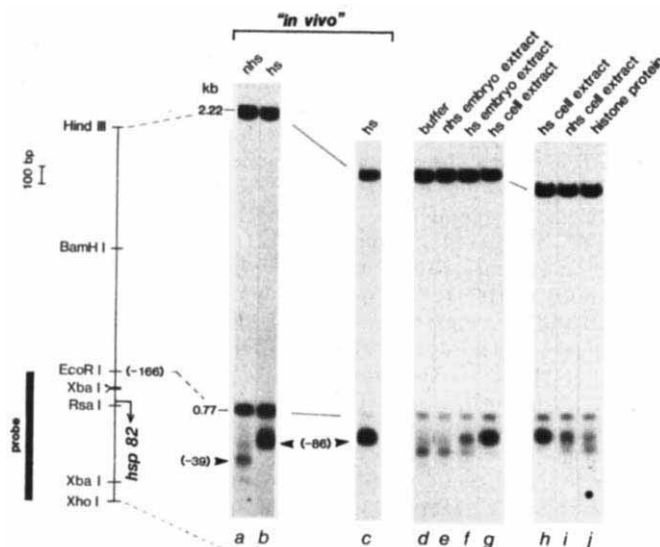


Fig. 2 Chromatin binding activity of extracted factors at the *hsp82* gene promoter. *a-c*, *exo*III digestion downstream from an *Eco*RI cut at position -166 in chromatin from non-heat-shocked (nhs) *Drosophila* embryos, and from embryos heat-shocked (hs) at 36 °C for 15 min. (+1) is the transcriptional start indicated on the partial restriction map by the arrow pointed in the direction of transcription. Arrowheads indicate the positions of the barriers to *exo*III digestion. Purified nuclei from 2-7-h-old *D. melanogaster* (Oregon R) embryos were digested with *Eco*RI and *exo*III; the DNA was purified, trimmed with *S*₁ nuclease, repurified, digested with *Hind*III and *Xho*I, electrophoresed, blotted to nitrocellulose and hybridized with an indirect end-labelling probe (solid bar) as described previously². *c*, From the same gel as *d-j*. *d-j*, *exo*III digestion from the *Eco*RI cut at position -166 in normal chromatin incubated with the indicated extracts and reagents.

Methods: Nuclei were purified from 2-7-h-old *Drosophila* embryos grown at 24 °C as previously described². The pelleted nuclei were resuspended in nuclear buffer² at 5×10^8 per ml, transferred to 1.5 ml Eppendorf tubes and centrifuged briefly in the microfuge. After removal of the supernatant, the pellet was resuspended in buffer alone (Solution III; see below), in extract plus 5 mM MgCl₂, or in 50 µg histone H1 protein (Boehringer-Mannheim) per ml of nuclear buffer, and incubated at room temperature, 24 °C, for 20 min. The nuclei were pelleted again and the supernatant was removed. Nuclei were then resuspended for digestion at 30 °C for 15 min in nuclear buffer—5% glycerol containing 3,500–6,100 U ml⁻¹ *Eco*RI and 9,000 U ml⁻¹ *E. coli* exonuclease III and MgCl₂ adjusted to 6 mM. The reactions were terminated with EDTA and SDS, and nucleic acids were purified by organic extraction and precipitated in EtOH. *S*₁ nuclease trimming of ssDNA tails was performed essentially as described², using 2,500 U ml⁻¹ *S*₁ nuclease for a DNA concentration of ~0.1 mg ml⁻¹. DNA was repurified and digested with *Hind*III and *Xho*I, electrophoresed on a 25 cm long 1.4% agarose Tris-acetate EDTA (TAE) gel, blotted to nitrocellulose and hybridized as above. Extracts were prepared using a modification of the procedure of Dignam *et al.*²¹. Schneider line 2 tissue culture cells were grown in suspension²² at 23–24 °C, to a density of 5×10^6 – 1×10^6 cells ml⁻¹. After washing in saline, pelleted cells were frozen in dry ice-EtOH and stored at -70 °C until use. Pellets containing about 10¹⁰ cells were thawed, and resuspended at 4 °C, the subsequent operating temperature, in two packed cell volumes of Solution I (0.3 M sucrose, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulphonyl fluoride (PMSF).) Cells were lysed in a Dounce homogenizer using about 15 strokes of the tight pestle. Breakage of cells was about 95% by microscopic inspection. The homogenate was centrifuged in a clinical centrifuge at top speed for 7.5 min (1,600g) to pellet crude nuclei. The nuclei were resuspended in 2 packed nuclei volumes of Solution II (10 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF) and the final NaCl concentration was re-adjusted to 0.4 M. The crude

nuclei were extracted at 4 °C for 30 min with continuous stirring, followed by centrifugation at 100,000g for 1 h. The supernatant was transferred into a colloidal bag (Schleicher and Schuell UH 100/10) for dialysis in Solution III (20 mM HEPES, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF) for about 5 h. A vacuum was applied for some time during dialysis to concentrate the extract to the original packed cell volume. The extract was cleared by centrifugation at 10,000g for 10 min. The supernatant (~50 mg protein per 10¹⁰ cells) was frozen in aliquots in liquid N₂, and stored at -70 °C; the extracts retained their activity for at least two months after storage. A 1/5 dilution of active extract still gave 'full' occupancy of the binding site, but further dilutions up to 1/40 reduced binding activity to undetectable levels. Embryonic extracts from 15 g each of normal and heat shocked (36 °C, 20 min) 0–18-h-old *Drosophila* embryos were prepared from nuclei purified by centrifugation through Solution I (1.7 M sucrose) as described previously² except that the homogenizing buffer was Solution I supplemented with leupeptin, 5 µg ml⁻¹, pepstatin, 2 µg ml⁻¹, and aprotinin, 0.2 TI U ml⁻¹. Embryonic nuclei were extracted for 30 min at 4 °C, with stirring, in 10 ml Solution II; NaCl was re-adjusted to 0.4 M to account for the volume of the purified nuclear pellet. The embryonic extract was centrifuged, dialysed, clarified and stored essentially as for extracts from cultured SL2 cells.



presumably due to high protease activity associated with the former. A non-heat-shocked SL2 cell extract also contains HAP binding activity (Fig. 2, lane *i*) (roughly 20-fold less than shocked cell extracts, as seen by dilution experiments, data not shown). This result is not unexpected as the *hsp82* gene is expressed at some level in *Drosophila* cell lines cultured under normal laboratory conditions^{6,9-11} and as HAP binding *in vivo* can be observed even in nonshocked SL2 cells (C.W., unpublished observations).

To map the proximal boundary of HAP binding *in vitro* we analysed the resistance to exonuclease digestion upstream from a *RsaI* cut at position +20 in the chromatin (Fig. 3). As shown previously², the weaker *in vivo* exonuclease resistance of the non-heat-shocked *hsp82* gene at position -17, the proximal border of the TAB binding site, is largely overcome in limit-digestion conditions, and the bulk of the *RsaI*-cleaved fragments are further degraded to a smear (not well reproduced in Fig. 3, lane *b*). On heat shock (lane *c*), HAP binding poses an impenetrable barrier at position -50, and also retards the kinetics of digestion through the TAB site. When non-heat-shocked nuclei are incubated with the indicated extracts, pelleted, and resuspended for digestion with *RsaI* and *exoIII*, the position of resistance to *exoIII* generated by *in vitro* HAP binding is identical to that shown *in vivo* (Fig. 3, lanes *d-i*). The specific variation in the binding activity of the extracts from embryos and cultured cells also parallels the analysis of exonuclease resistance from the *EcoRI* cut in Fig. 2.

We can assay for this binding independently by measuring the sensitivity to *XbaI* cleavage in chromatin at two sites positioned at -74 and -64, within the HAP binding site. When

non-heat-shocked embryonic nuclei are incubated with the extract from heat-shocked SL2 cells, the *XbaI* sites at positions -74 and -64 are clearly protected from cleavage (Fig. 3, lane *m*). Interestingly, the other *XbaI* site at position +484, inside the *hsp82* transcriptional unit, does not become more sensitive upon shock as it does *in vivo*, suggesting that the extract may be unable to effect the secondary phenomenon associated with chromatin activation.

The chromatin binding activity is unaffected by treatment of active extract with RNase A + TI, or with micrococcal nuclease. However, when extracts are treated with trypsin or proteinase K (subsequently inactivated with protease inhibitors), HAP binding activity is abolished using the exonuclease protection

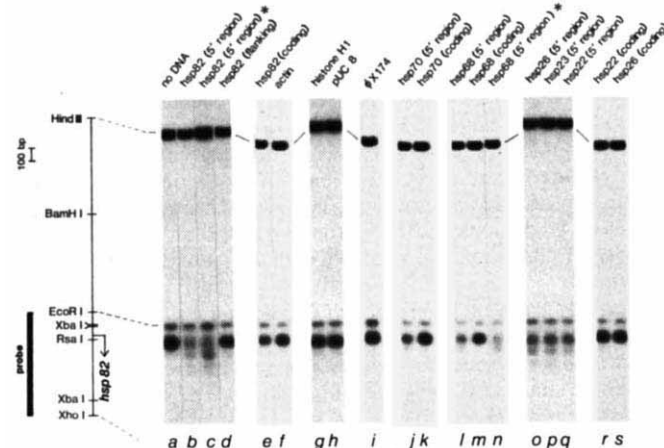


Fig. 4 DNA inhibition of HAP binding to *hsp82* gene chromatin. Extracts from heat shocked SL2 cells were diluted 5-fold; 100 μ l was incubated with the indicated DNA's for 10 min at 24°C, followed by incubation with nuclei purified from nonshocked *Drosophila* embryos. The DNA templates were linearized by restriction cleavage before incubation. The inhibition produced by different parts of each heat shock gene is compared using approximately equivalent DNA concentrations for the particular sequence. Specific chromatin-binding activity was assayed by exonuclease protection as in Fig. 2 legend. *b*, *hsp82* (5' region); 0.05 μ g of 186 bp, *EcoRI* (-166) to *RsaI* (+20) fragment, with 0.95 μ g of ϕ X174-*HaeIII* as carrier DNA. *c*, DNA as in (*b*) was incubated with extract as above. *CaCl*₂ was added to 0.4 mM, micrococcal nuclease to 300 U ml⁻¹ and free DNA was digested for 5 min at 24°C. Micrococcal nuclease was inactivated with EGTA to 1 mM and the extract was then incubated with noninduced nuclei as above. *d*, *hsp82* (flanking); 0.2 μ g of 0.7 kb upstream *BamHI* to *EcoRI* (-166) fragment with carrier DNA as above. *e*, *hsp82* (coding); 0.25 μ g of 1.8 kb *XhoI* (+584) to *SalI* (+2,400) fragment with carrier DNA as above. *f*, Actin gene; 1 μ g of linearized plasmid DNA consisting of pBR322 vector and a 3.8 kb *BamHI*-*EcoRI* insert containing coding and upstream sequences for the *Drosophila* actin gene at chromosomal locus 57A (E. Fryberg, personal communication). *g*, Histone H1 gene; 0.75 μ g of a 1.3 kb *BamHI*-*HpaI* fragment containing roughly the 5' half of the coding sequence plus upstream DNA²³. *h*, pUC8; 1 μ g of the linearized plasmid DNA. *i*, ϕ X174; 1 μ g of duplex phage DNA cut with *HaeIII*. *j*, *hsp70* (5' region); 0.4 μ g of 279 bp *XhoI* (-194) to *PstI* (+85) fragment. *k*, *hsp70* (coding); 1 μ g of 1.1 kb *PstI* (+85) to *PstI* (+1,187) fragment. *l*, *hsp68* (5' region); 1 μ g of 0.4 kb upstream *PstI* to *HpaII* (+44) fragment²⁴. *m*, *hsp68* (coding); 1 μ g of 0.6 kb *PstI*-*PstI* fragment of coding sequence²⁴. *n*, 1 μ g of *hsp68* 0.4 kb 5' *PstI*-*HpaII* fragment was incubated with extract, digested with micrococcal nuclease and processed as in (*c*). *o*, *hsp26* (5' region); 1 μ g of linearized plasmid DNA consisting of pUC9 vector and a 0.7 kb upstream *PstI*-*EcoRI* (+7) fragment²⁵⁻²⁸. *p*, *hsp23* (5' region); 1 μ g of linearized plasmid DNA consisting of pUC9 vector and a 0.7 kb upstream *PstI*-*EcoRI* (+7) fragment²⁵⁻²⁸. *q*, *hsp22* (5' region); 1 μ g of linearized plasmid DNA consisting of pUC9 vector and a 0.6 kb *HindIII* (-402) to *HindIII* (-213) fragment²⁵⁻²⁸. *r*, *hsp22* (coding); 1 μ g of linearized plasmid DNA consisting of pUC9 vector and a 0.8 kb downstream *BamHI* to *HindIII* (+213) fragment²³⁻²⁸. *s*, *hsp26* (coding); 1 μ g of linearized plasmid DNA consisting of pUC9 vector and a 0.6 kb downstream *PvuII* to *EcoRI* (+7) fragment²⁵⁻²⁸.

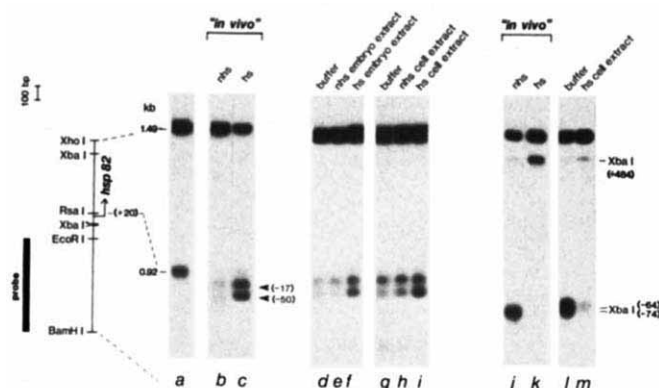


Fig. 3 Proximal boundary of HAP factor binding *in vitro*. *b*, *c*, *exoIII* digestion upstream from an *RsaI* cut at position +20 in chromatin from nonshocked embryos (*b*), and from embryos heat shocked at 36°C for 15 min, (*c*). Arrowheads indicate the barriers to *exoIII* digestion. Purified nuclei were digested with *RsaI* and *exoIII*; the DNA was purified, trimmed with *S*₁ nuclease, repurified, digested with *BamHI* and *XhoI*, electrophoresed, blotted to nitrocellulose and hybridized with labelled probe (solid bar). The position of the initial *RsaI* cut is shown in *a*. *d-i*, *exoIII* digestion from the *RsaI* cut at position +20 in normal chromatin preincubated with extract or buffer alone. Nuclei treated with extract or buffer as in Fig. 2 legend were resuspended for digestion at 35°C for 12 min in nuclear buffer-5% glycerol containing 1,300 U ml⁻¹ *RsaI* plus 9,000 U ml⁻¹ *exoIII* (*d-f*), or 2,300 U ml⁻¹ *RsaI* plus 9,000 U ml⁻¹ *exoIII* (*g-i*). The reactions were stopped, the purified DNA treated with *S*₁ nuclease, repurified, restricted with *BamHI* and *XhoI*, electrophoresed on a 40-cm-long TAE agarose gel, blotted to nitrocellulose and hybridized as above. *j-m*, *XbaI* sensitivity of the *hsp82* gene at positions -64, -74 and +484 in chromatin from nonshocked embryos (*j*), from embryos heat shocked at 36°C for 15 min (*k*), and in non-shocked embryonic chromatin incubated with buffer alone (*l*), or with an extract made from heat shocked SL2 cells (*m*). Nuclei purified from 2-7-h-old *Drosophila* embryos were digested with 1,400 U ml⁻¹ *XbaI* at 35°C for 15 min (*j*, *k*), and with 1,700 U ml⁻¹ *XbaI* at 35°C for 12 min for the nuclei incubated with buffer or extract (*l*, *m*). DNA was purified and restricted for blot analysis as above.

assay as above (data not shown). This confirms that the specific binding component of HAP is protein.

Specific HAP binding to *hsp82* gene chromatin is almost totally inhibited by pretreating active extracts with a short *hsp82* DNA fragment containing the binding site (Fig. 4, lane *b*). No inhibition on pretreatment is observed using further upstream flanking sequences or sequences in the *hsp82* coding region, nor when other DNAs are used (lanes *d-i*). On the other hand, purified DNA from the 5' region of the other heat shock genes: *hsp70*, *hsp68*, *hsp26*, *hsp23*, *hsp22* do inhibit to a partial extent the binding of HAP factor to *hsp82* gene chromatin (lanes *j, l, o-q*), whereas purified DNA from the coding sequences of *hsp70*, *hsp68*, *hsp22* and *hsp26* do not (lanes *k, m, r, s*). To demonstrate that the HAP activity we have measured in the extract is a primary DNA binding activity and not a secondary enzymatic activity which converts an ineffective HAP to its specific DNA binding mode, we pretreated active extract with target DNA and digested excess DNA with micrococcal nuclease. The resultant extract has no HAP binding activity towards the *hsp82* gene chromatin (Fig. 4, lanes *c, n*); the activity should have been retained were the active component secondary to DNA binding. (Effective removal of excess DNA by micrococcal nuclease is demonstrated by the recovery of HAP binding activity to chromatin on readdition of active extract; data not shown.)

We infer from these inhibition experiments that HAP factor for the *hsp82* gene is able to bind to purified DNA, not only to its own target sequence but also to the upstream consensus sequence³ shared by other heat shock genes. The simplest model consistent with the data is that HAP, in concert with bound TAB, can coordinately activate the whole heat shock gene family by binding to each upstream element, and that the variation in the kinetics of activation among these genes reflects differential binding affinities of HAP for individual upstream elements that are similar but not identical in sequence. Such a model would predict a direct relationship between decreasing HAP binding affinities and the increasing order of transcriptional activation. The available evidence supports this prediction. The *hsp82* gene is most easily activated^{6,9-11,12}, whilst HAP binding strength *in vivo* (ref. 2 and unpublished data), or *in vitro* (Fig. 4) is greater for the *hsp82* gene than for other heat shock genes. It will be of interest to investigate further the correlation between binding strength and activation kinetics.

We re-examined the sequence of the 36 bp HAP binding site² for *hsp82* and found that it covers a central 28 base sequence with almost perfect dyad symmetry (24 bases out of 28): (–81) GAAGCCTCTAGAAG|TTTCTAGAGACTTC (–54). None of the upstream elements of the other heat shock genes have this high degree of symmetry. The exceptionally strong binding affinity of HAP to this symmetrical dyad suggests that it may be the canonical recognition sequence for HAP binding (as a protein dimer or tetramer like prokaryotic DNA binding proteins).

We do not believe that HAP is synthesized *de novo* upon heat shock, because the response can be brought on within minutes after stimulation, and can occur in the absence of protein synthesis¹³. It seems that HAP should either be decompartmentalized upon heat shock or more likely, be converted from a pre-existing inactive form to a specific DNA binding form. Our finding that HAP activity in the crude extract binds DNA directly does not preclude the presence of secondary modifying activities in the extract. We are currently devising appropriate assays to retrace the physiological pathway from the terminal events of heat shock.

Others have shown the presence of eukaryotic sequence-specific DNA binding proteins^{14,15}, and activators of heat shock genes have been reported in studies employing different methods¹⁶⁻¹⁹. HAP factor appears to be unrelated to these activities. During the preparation of this manuscript, Parker and Topol²⁰ published a report on a *Drosophila* transcription factor designated HSTF that is specific for the *hsp70* gene; partially purified preparations of this factor bind to a 55 bp region

upstream of the TATA box. HAP and HSTF bind roughly to the same DNA sequence, but their binding activities in non-heat-shocked cells appear to be very different; further work should illuminate the relationship between the two factors.

We believe that the *in vitro* chromatin binding, exonuclease protection assay reported here is a powerful method for analysing eukaryotic DNA binding proteins for the following reasons: (1) precision in delimiting the binding site, (2) confidence in the physiological relevance of the binding provided by *in vivo* comparison, and (3) exceptional sensitivity, as seen by detection of binding activity in crude extracts with no fractionation whatsoever. This feature permits binding activity to be tracked from the start of the purification; at later stages, more traditional assays could be used to increase experimental efficiency.

I thank B. Sauer, C. Klee, P. Wagner and S. Wilson for helpful suggestions on protein analysis, E. Craig, E. Fyrberg, R. Holmgren, R. Blackman, R. Morimoto and M. Meselson for gifts of cloned DNA, T. Paisley for technical assistance and B. Miller for typing the manuscript.

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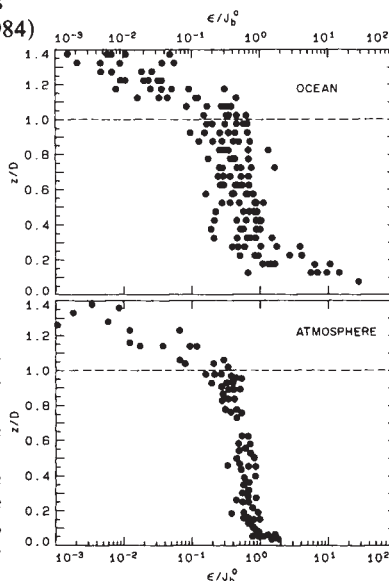
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Turbulence in an oceanic convective mixed layer—Corrigendum

T. J. Shay & M. C. Greg

Nature **310**, 282–285 (1984)

In the above letter it was stated that the mixed layer mean value of ϵ/J_b^0 in the ocean was a factor of two lower than that in the atmosphere, and that difference was left unexplained. The difference was the result of a computational error. When that error is corrected, the two cases are in much better agreement, as seen in the figure. For the region $0.4 < z/D < 1.0$, the mean value of ϵ/J_b^0 in both the ocean and atmosphere is 0.45. The difference in scatter may be due to more restrictive editing of the atmospheric data as well as the influence of lateral processes in the ocean.



Theology *in vitro*

John Habgood

Begotten or Made? By Oliver O'Donovan.
Clarendon: 1984. Pp.88. Pbk £2.50, \$4.95.

THE debate sparked off by the Warnock Committee report on human fertilization should benefit from this unusual contribution by Professor O'Donovan. As befits the Regius Professor of Moral and Pastoral Theology in the University of Oxford he approaches the topic at a high level of generality, in sharp contrast to the pragmatic and piecemeal procedure often favoured by practising scientists. He modestly claims to make no more than a contribution to the discussion, but his main argument, if accepted, would radically change the thrust of much current practice in this field. I hope that scientific readers who find it hard to cope with his style of argument, and in particular with his Christian presuppositions, will nevertheless persevere with the book if only for the sake of the fresh perspective it opens up.

One of the discouraging and perplexing features of many of the moral choices facing medical practitioners is not the fact that difficult decisions have to be made, but the sense that medical practice is part of a vast and seemingly inevitable process of social change. It is the pre-emption of choice which is worrying, and this is why no amount of analysis of particular cases, and no attempts to specify legal limits in relation to this or that technique, can fully resolve the underlying dilemmas. Effective moral comment entails criticism of the cultural assumptions which make it easy and natural to look for one kind of solution to our practical problems in preference to another. Only by tackling the issues at this level is it possible to see, and maybe begin to counteract, the full implications of runaway technology.

The impact of living in a technological culture carries particular threats for the process of human begetting. What distinguishes our relationship with human beings in such an environment is precisely the fact that they are not "made".

A being who is the 'maker' of any other being is alienated from that which he has made, transcending it by his will and acting as the law of its being. To speak of 'begetting' is to speak of quite another possibility than this: the possibility that one may form another being who will share one's own nature, and with whom one will enjoy a fellowship based on radical equality [p.2].

It is this radical equality, this deep set of assumptions about the status and being of persons, which Professor O'Donovan believes is eroded by reproductive techniques which break up the act of begetting into a series of manipulations. If it is objected that science and medicine are doing no

more than to supplement natural processes which have gone wrong, then the reply is that it is precisely this assumption — that every human ill has a technological answer — which in the end threatens our humanity.

The theme is illustrated by reference to trans-sexual surgery, artificial insemination by donor, experimentation on embryos and *in vitro* fertilization. The first of these raises important questions about the degree to which social perceptions of a person's sex can be used to justify surgical interference with the given characteristics of their body. The second highlights the erosion and confusion of the role of parents. The third leads to an illuminating discussion of the meaning of the word "person", and the tendency to convert a concept whose primary reference is ontological and historical into a qualitative term, indicating the possession of a particular quality, "personality". Embryos, on this latter view, do not yet possess personality and so may be experimented on as being interestingly human but not yet persons to be respected. O'Donovan comments,

The practice of producing embryos by IVF with the intention of exploiting their special status for use in research is the clearest possible demonstration of the principle that when we start making human beings we necessarily stop loving them; that that which is made rather than begotten becomes something that we have at our disposal...[p.65].

On IVF itself he accepts the arguments for "assisted reproduction", but wonders whether the price in terms of research and risk-taking is morally too high.

This is not a book which will please those who follow their scientific and technological noses wherever the evidence and opportunities lead them. Even a sympathetic reader like myself spattered the margins with question-marks. Its merit is that it raises some fundamental issues about the character of the civilization we are making (the word is significant) for ourselves, in a way which gives current discussions of particular techniques a new sense of urgency. The fact that its title echoes a phrase in one of the Christian creeds, referring to Christ as "begotten not made", is a reminder of the extent to which traditional Western assumptions about the nature and value of human life have been rooted in Christian theology. I suspect that theology still provides the best defence against the dehumanizing tendencies of a technological culture, but it has to be admitted that, humanly speaking, the defence looks uncomfortably fragile. □

John Habgood is Archbishop of York.

The orderly chromosome

J.R.S. Fincham

Molecular Evolution and Organization of the Chromosome.

By A. Lima-de-Faria.

Elsevier Science: 1984. Pp.1, 186. Dfl. 450, \$191.50.

THE scope of this book is broader than its title might suggest; it provides a reappraisal of genetics and a very individual view of life. Its main weight lies in an extensive review of many aspects of chromosome behaviour, especially those involving interactions between different parts of genomes. The author has for many years promoted the idea of the "chromosome field", the essence of which is that the arrangement of sequences within chromosomes matters for function. There is therefore much emphasis on such topics as *Drosophila* variegation-type position effects, control of the activity of nucleolus-organizer regions, the activity of centromeres and neocentromeres, and inter-chromosomal effects on crossover frequency. None of these has yet been explained in molecular terms, but the

author is quite clear that they are molecular phenomena.

In his preface, Professor Lima-de-Faria remarks that his book is mainly based on the 25,000 reprints in his personal collection, supplemented by over 3,000 photocopies. Many of these have been drawn upon to supply the book's copious tables and figures which, for the most part, appear with legends reproduced verbatim from the originals. This does not always conduce to close integration with the text but at least helps, I suppose, to minimize error. In preparing a book of the magnitude, it is obviously difficult to be up-to-the-minute in every area; of the (approximately) 2,000 references in fact fewer than 10 per cent date from within the past five years. Thus we find only a brief mention of the nucleosome and little or nothing on non-histone proteins, the sequences of eukaryotic promoters and enhancers, methylation and the relationships of all these to states of transcriptional activity and inactivity.

It is clear that Lima-de-Faria's object in writing this book was not only to review a field but also to correct a number of what he sees as misconceived notions in modern biology. In nuclear cytology he considers that much harm has been done by the concept of chromosome *mechanics*. He

looks to molecular rather than mechanical explanations of chromosome behaviour, and evidently sees a conflict between the two. More generally, he is in profound opposition to the idea of randomness, and upholds the principle of order in all things.

This attitude emerges in a number of striking passages. Discussing meiosis (p. 923), he dismisses the view that disjunction of different bivalents occurs at random, making the point that, if this were so, there would be nothing to prevent whole parental chromosome sets from segregating out together. Rather, he says, the bivalents are *directed* to segregate independently of one another. Earlier in the book (p. 415) he criticizes Ohno for his speculation that many segmental rearrangements have been fixed in evolution by chance, being in themselves neutral or "harmless". Lima-de-Faria maintains that to say that a rearrangement is harmless is to imply that there is an organization in the chromosome that could in principle be harmed and so Ohno is contradicting himself — a formidable logical trap indeed! Naturally, Lima-de-Faria has no time for the concept of "junk" DNA, disposing of it with another brief argument (p. 1,030). When one actually looks at such a piece of putative junk, he says, one finds that it has a definite sequence and definite locations in the genome — it clearly is not accidental at all! It is not surprising to find him also opposed to the idea of mutations occurring at random; he points out that they are now known to have causes, including that new class of directive agents the transposable elements. Indeed, nothing is accidental in Lima-de-Faria's view of the world. Everything is determined by the laws of physics and chemistry.

As a corollary of his belief of physico-chemical determinism, Lima-de-Faria considers that natural selection is a greatly over-rated concept. "Selection", he says (p. 147), "appears to be impotent in producing major changes in the face of the molecular determination locked in the chromosome and the cell". As a means of explaining evolution, natural selection is largely redundant in any case, since there is not that much evolution to explain. Organisms use their genes in so many ways that they do not need to have many of them, and most of those that they do have they have in common. Thus (pp. 1,060) the bee orchid is able to imitate the bee, and a grasshopper is able to camouflage itself as a leaf, because insects and plants share many of their genes!

Those who have access to this book will find it useful in at least two ways. First as an extensive and generally interesting collection of snippets from Professor Lima-de-Faria's reprint library, second as a rich source of quotations suitable for use as provocative essay topics for first-year students. □

J.R.S. Fincham is Balfour Professor of Genetics at the University of Cambridge.

China under the plough

Charles Higham

Science and Civilisation in China. Volume 6, Part II Agriculture.

By Francesca Bray.

Cambridge University Press: 1984.

Pp. 724, £50, \$99.50.

THE vastness and longevity of Chinese civilization are the joint themes of one of the great scholarly adventures of the century: Joseph Needham's *Science and Civilisation in China*. Even the most casual acquaintance with the successive volumes will rapidly dispel any ethnocentric smugness on the part of the Westerner in the priority or superiority of his Graeco-Roman heritage. For the sinophile, each new volume is, by definition, the basic reference in English. The latest to appear is arresting not only because it is the first to be written by one of Needham's research associates, but also because its theme, agriculture, is basic to any appreciation of the productive forces which underwrote Chinese civilization.

Let it be said that this is a massive compilation: the references alone run to 50 crowded pages, and 38 are necessary to contain the index. The preceding text follows an almost military step. The first section stresses China's geographic and climatic diversity, setting the stage for the subsequent contrast between the dryland systems of the north and the wet rice cultivation which characterized the warmer terrain south of the Yangtze. We are then introduced to the source materials, their potential and deficiencies. Literary sources begin with the Shang oracle texts and proceed through three-and-a-half millennia of expansion, addition and recension. The growing body of archaeological evidence illuminates the earlier half of the history of Chinese agriculture, and supplements the historic sources for the later phases.

The main empirical thrust is reserved for a detailed and analytical review of cultivation systems, land tenure, classes of tools, crops and techniques of soil preparation, husbandry and, of course, harvesting, processing and storage. Apparent blind spots, such as the methods of water control, have been considered in earlier volumes or are promised for later ones.

It could be argued that the vast array of facts would have revealed more structure had the author treated her subject holistically, reviewing, for example, first the pre-historic and then the dynastic phases sequentially rather than taking specific implements or techniques and following their development through the millennia. The non-specialist must be nimble-minded to be aware, all the time, where he is

chronologically. This possible source of confusion is not mitigated by the illustrative material. It is true that many of the original Chinese drawings of agricultural scenes are charming and highly informative. They would have been more so had the author placed a date or at least a dynasty in the caption. Many of the author's illustrations are most uneven in quality; many also lack a scale, and those scales which are provided vary between the imperial and the metric.

Yet it must be remembered that this is but one of a series of volumes which aims to detail Chinese achievements in all fields, blow by blow. It is not, by definition, an entity with the aim of synthesizing Chinese agriculture within its social and technological contexts. It is, therefore, refreshing to find that we are not denied some contextual analyses.

Agriculture is the engine which sustains statehood. The polity out of step with its agricultural system will soon trip. Through the millennia of population growth, territorial expansion and external threat, the Chinese farmer has displayed a necessary blend of innovation and conservatism. We are invited to consider three instances of the articulation between society and subsistence.

The first concerns agriculture under the Shang. Shang civilization is regarded as the earliest complex society in China, flourishing for five centuries from c. 1500 BC. The basic issue is how the Shang rulers, bureaucrats and craft specialists were sustained in such centres as An-Yang. The agricultural techniques of the Shang peasantry are hardly known: archaeologists have concentrated on royal tombs and elite residences, and oracle bones are not concerned with the minutiae of agriculture. Bray favours a system based on plough cultivation. Now the plough is a very important implement. Goody, whose basic text on the social implications of ploughing is absent from the bibliography, has stressed the adhesions which exist between the extra productive capacity of ploughing and social organization, generation of surplus and the organization of marriage and inheritance. Archaeological sources are silent on the issue of Shang ploughing, however, and it could be argued that Bray has tried unduly hard to extract the necessary evidence. For example she is in error when describing the bronze ploughshares from Tonkin as being contemporary with Shang. Vietnamese archaeologists are unanimous in ascribing them to the Dong Son culture, which is a millennium later. Again, Wheatley's view is that the Shang centres were more ceremonial and elitist than centres of dense population. If he is right, then the need to invoke ploughing to produce large food surpluses is reduced.

The author is more assured when handling documentary sources. As a second example, we are given a revealing glimpse of the changing basis of land

tenure under the Han — the state-sponsored aid to improve output provides so many parallels with recent Western attempts that one needs reminding that Han events took place 2,000 years since.

During the Sung the political centre of China moved south to the wet rice lands, and this introduces Bray's third instance of the articulation between social and agricultural variables. The wet rice agricultural technique involves parcelling the land into level plots separated by bunds. The soil in these fields — which are always small and often minute — is turned by the plough, then converted by harrowing to a creamy consistency prior to the transplanting of rice from a seed bed. The labour input during the planting and harvesting seasons is very high, and the regulation of water flow during the growing season is crucial. During the off-season, however, peasants may turn to cottage industry or, given the surpluses produced by farmers, some may specialize in craft production or exchange.

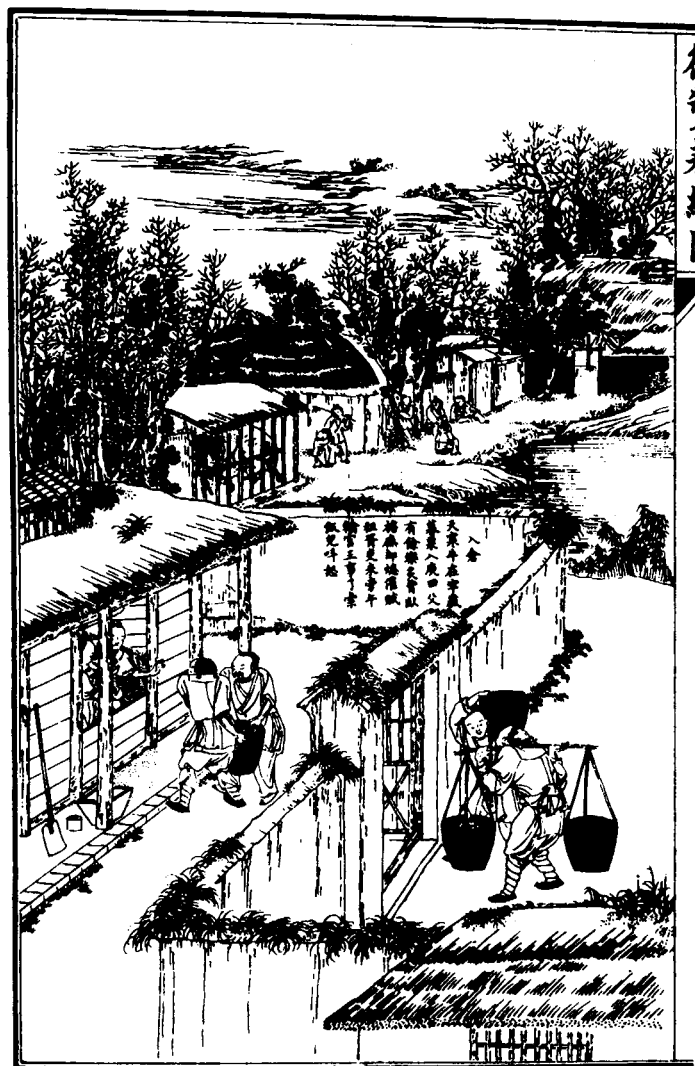
A basic tenet of this work is that such an essentially small-scale individual or family system is not susceptible to the revolutionary changes in productivity and intensification which characterized Europe's industrial revolution. So we encounter an

intriguing paradox: growing European familiarity with Chinese agricultural technology, particularly the efficient curved metal mouldboard and the multiple seed drill, had a revolutionary impact when applied to European agriculture. In China, however, intensification in the face of a rising population involved not mechanization, but subtle refinements in wet rice agriculture which, of its very nature, failed to enter into a multiplier effect with the social or technological systems. This interplay did not stop with Mao's revolution: in effect modern production teams are a close image of the Sung communal unit, the backbone of wet rice cultivation.

The debate which such hypotheses will surely stimulate requires the full availability of data, an axiom which has been scrupulously adhered to. The portrayal of Chinese agriculture requires the broad brush of generality and the miniaturist's stroke of critical detail. This monumental and important book has both in full measure. □

Charles Higham is Professor of Anthropology at the University of Otago, New Zealand.

The illustration is taken from *Science and Civilisation in China* depicting a thatched village granary.



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Out of the blue

Derek W.G. Sears

Chondrules and Their Origins.

Edited by Elbert A. King.

Lunar and Planetary Institute, Houston: 1984. Pp.377. \$33.

CHONDRULES are millimetre-sized silicate assemblages found in meteorites and, like their hosts, they have a unique story to tell about the conditions in the early Solar System. Hitherto, information about them has been spread through a vast literature. So an entire book devoted to the topic is long overdue.

Chondrules and their Origins is essentially the proceedings of a two-day workshop held at the Lunar and Planetary Institute, Houston, in November 1982. It suffers all the strengths and weaknesses typical of such books. The contributed papers are highly variable in approach and quality — some are significant new contributions, a few are useful reviews, and some are brief re-statements of particular views — and the coverage of the subject is rather patchy. Several prominent contributors to the field are conspicuous by their absence and, while petrologic studies abound, chemical and isotopic papers are under-represented; of the 25 papers included, 14 deal with petrology, 3 with chemistry and 3 with isotopic studies (of which only one is on radiometry). There are also 5 which deal with model-making, qualitative and quantitative.

Examples of the useful reviews are the summary of radiometric work by Swindle, Caffee and Hohenberg, who conclude that the chondrule-forming epoch was brief and occurred at the time of the formation of the meteorites (and the Solar System), and the masterly synthesis of compositional data by Grossman and Wasson. The latter authors argue for a view that has become popular within the past few years, namely that chondrules were formed by the melting of pre-existing, fine-grained dust. The once-popular idea that chondrules could form directly by condensation from the nebular gas has almost no supporters now, although Blander advocates a modification of the idea. In a somewhat more narrowly-

focused, but equally worthwhile review, Taylor, Scott and Keil amass arguments against the idea that chondrules could form by impact, volcanism or accretional heating.

One notable example of an original contribution is the paper by Nagahara, who describes another major line of evidence that chondrules were made by melting pre-existing dust; she describes several grains which survived melting. Another is the article by R.N. Clayton and co-workers; on the basis of oxygen isotope data, they argue that although the chondrules from different classes of meteorite formed from different solid material, they were melted in a common gas. There are also two brief but fascinating accounts of astronomical data relevant to chondrule formation by D.D. Clayton and D. Heymann.

In his foreword, Elbert King, the editor,

states that his aim was to provide a broad overview of current ideas. I am not convinced that the book succeeds totally, being top-heavy in specialized contributions and leaving certain areas devoid of attention. For example, a review of ideas on the heat-source which melted the chondrules would have been welcome. The book might also have come closer to achieving its aims had it been organized differently — the sequence of papers, alphabetical by first author, is probably the least helpful possible. Surely it would have been better to have gathered the papers by subject area, with each section being led by a review. Still, the book has served as a vehicle for many valuable papers which might not otherwise have been written, and for that Elbert King is to be congratulated.

Derek W.G. Sears is a Professor of Chemistry at the University of Arkansas, Fayetteville.

Amphibian's book of lists

Jonathan Slack

Amphibian Morphogenesis.

By Harold Fox

Humana: 1984. Pp.301. \$59.50 (US), \$69.50 (elsewhere).

TABLES of standard stages enable embryologists to know precisely at which point in development their experiments have been conducted and to communicate this information to others. But anyone who has worked with amphibian embryos probably feels that stage series are somewhat arbitrary and do not capture the true intrinsic periodization of development. Embryos seem to spend hours at stage 9 (late blastula) and then gallop through gastrulation to stage 13. There they wait for even longer and then race even faster through neurulation to stage 20. Presumably the springs which drive the morphogenetic movements are being coiled at stage 9 and stage 13 and the subsequent events represent the mechanical consequences.

Amphibian Morphogenesis must be the first book to list stage series for 192 species of amphibia, a more or less comprehensive collection. Alas it is just a list. None of the potentially interesting features of stage series are critically discussed, for example the question of intrinsic periodization or the extent to which the timing of one event can be altered relative to another in evolution. The list-like quality pervades the whole book and makes one aware of the difference in approach to a problem adopted by biomedical scientists on the one hand, who are chiefly interested in developmental mechanisms and regard amphibia as useful models for experimental work, and zoologists on the other who would like to catalogue the anatomy,

physiology, ecology and evolutionary history of all 2,850 amphibian species.

This book will probably fail to satisfy either audience. Its core is Section III which reviews organ development in larvae. This is the best part and although it is dull reading it will be useful in laboratories such as mine where occasionally we suddenly want to know all about the development of the lateral line, the cement gland or the blood. It contains a useful collection of references but would have benefited from more diagrams and more light microscope photographs. It is full of electron micrographs but these are relatively uninformative because the magnification is too great to show the specific features of the tissues.

There are three other sections in the book, dealing with evolution (I), stage series (II) and embryogenesis (IV). Curiously enough, on close inspection, all of them seem to be mainly about hormones and metamorphosis just like Section III. The full and meticulous references to metamorphosis contrast strangely with the casual treatment of other subjects; for example on p. 4 we read that

During the warm, moist period of the Carboniferous era, the earth was covered by swampy forests and giant ferns, which later fossilized into coal. Conditions were favorable for amphibian development and a great variety of them evolved in this great age of the "Amphibia," for they required warmth to maintain a high enough body temperature, the prevailing moist atmosphere hindered desiccation and food was plentiful.

We must conclude that this is essentially a review article about metamorphosis onto which has been grafted some rather subjective passages about other aspects of amphibian biology. Only those with a passionate interest in metamorphosis are likely to be tempted to acquire it. □

Jonathan Slack is the Head of the Experimental Morphology Laboratory at the Imperial Cancer Research Fund Laboratory, Mill Hill, London.

New in paperback

●*Plurality of Worlds: The Origins of the Extraterrestrial Life Debate from Democritus to Kant*, by Steven J. Dick. Publisher is Cambridge University Press, price is £7.50, \$12.95. For review see *Nature* 298, 588; 1982.

●*Endangered Lives: Public Health in Victorian Britain* by Anthony S. Wohl. Publisher is Methuen/Harvard University Press, price is £7.95, \$7.95. For review see *Nature* 305, 164; 1983.

New at the Boston show

The products covered this week include some on view at Nature's Boston conference, and some recent introductions in the field of biotechnology.

● A new C-tailing and cloning system intended to bring convenience and reproducibility to the construction of DNA recombinants, capable of transforming *Escherichia coli* cells at high levels of efficiency, has been introduced by New England Nuclear (NEN). NEN's new C-tailing and cloning system is self-contained and provides an oligo (dG)-tailed cloning vector, all critical chemical reagents, ready to use competent cells, control DNAs and procedures for successful, reproducible incorporation of DNA into the *E. coli*-pBR322 host-vector system. The method can be used to clone any double-stranded DNA, but is especially useful in cloning cDNA and DNA not suited to cloning by other methods. The results provided with the kit confirm that a control *Hae*III digest of M13mp8 DNA receives uniform, compact C-tail distribution in the biologically active range (15–25 residues). And, following annealing of the C-tailed digest to the system's oligo (dG) tailed pBR322 vector, transformation levels in competent cells are between 2×10^5 and 1×10^6 transformants per μg vector.

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● Triton Biosciences Inc. now manufacture affinity purified antibodies to an oncogene protein and a transforming growth factor. Each antibody is generated against a synthetic peptide corresponding to an amino acid sequence in the native protein. The antibodies are purified by affinity chromatography, and Western blot and immunoprecipitation analyses are used to verify reactivity and specificity. Anti-ras^Hp21 antibody is the first in a new series of oncogene analysis products from Triton. Ultimately such antibodies could be used in diagnostic tests to detect human malignancies.

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● New England Biolabs has developed an *in vitro* method for creating novel DNA cleavage specificities by using DNA methylases in conjunction with restriction endonucleases as a valuable new tool for gene cloning and DNA sequence analysis. Added to the New England list of 78 restriction endonucleases are: *Dpn*I (GmA/TC), *Xho*II (Pu/GATCp), *Sna*BI (TAC/GTA), *Hin*PI (G/CGC), *Nla*III (CATG/), *Nla*IV (GGN/NCC), *Ssp*I (AAT/ATT), *Spe*I (A/CTAGT), *Nhe*I (G/CTAGC), *Nsi*I (ATGCA/T) (isoschizomer of *Ava*III), and *Dra*I (TTT/AAA) (isoschizomer of *Aha*III). New DNA methylases include: *M. Alu*I, *M. Cla*I, *M. Tag*I, *M. Hph*I and *Dam*. New M13 cloning vectors now available are: M13mp10w RF I DNA, M13mp11w RF I DNA, M13mp18w RF I DNA and M13mp19w RF I DNA. Among the new products from the range of New England Biolabs' Organic Synthesis Division are the following phosphorylated linkers: *pXba*I, *d(pCTCTAGAG)* and *pBcl*II, *d(pCTGATCAG)*.

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● A new product data sheet from Schleicher & Schuell gives specifications and selection guidelines for S&S pure NC nitrocellulose, the standard immobilization matrix in nucleic acid and protein research. S&S nitrocellulose is highly selective and offers high binding capacity, low background radiation, and lot-to-lot consistency. S&S NC is suitable for the transfer of RNA, DNA, and protein, capillary and electrophoretic transfers as well as plaque and colony hybridization studies. It can also be used in trichloroacetic acid precipitations and dissolved for scintillation counting. The illustrated fact sheet includes a selection guide for choosing the most suitable dimension, pore size, and format. S&S pure nitrocellulose is available in a wide variety of sizes of circles, squares, sheets, and rolls, with special grid patterns, and in pore sizes ranging from $0.45 \mu\text{m}$ to $0.025 \mu\text{m}$.

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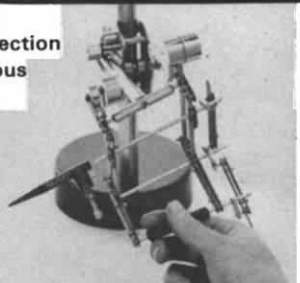
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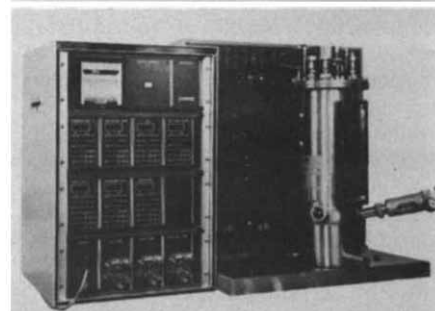
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The RF-1 incubation system from Bioengineering Associates (top) and New Brunswick's fermentation controller.

● Two full-range, hand-held pH meters with clear digital displays have recently been introduced by Corning. Constructed to cope with indoor and outdoor working environments, the new meters — Models 103 and 105 — can be used with either remote or clip-on electrodes and offer a resolution of 0.01 pH units. The meters can also be used for ion selective electrode work.

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